Natural Variation in Lifespan and Stress Responses in *Caenorhabditis elegans*

by

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"What would you attempt to do if you knew you cannot fail?"

-Unknown-

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List of Abbreviations

QTL	Quantitative Trait Loci
RILs	Recombinant Inbred Lines
ILs	Isogenic Lines
DR	Dietary Restriction
mTOR	Mechanistic Target of Rapamycin
PKA	Protein Kinase A
Ras	A membrane-associated guanine nucleotide-binding protein
N2	Collected from Bristol, most commonly used strain of *C. elegans*
BO	Collected from France, Bergerac strain is highly unstable
LOD	Logarithm Of the Odds
SNP	Single Nucleotide Polymorphism
AFLP	Amplified Fragment Length Polymorphisms
eQTL	Expression Quantitative Trait Loci

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Abstract

Wild animals are adapted to survive in different niches and therefore represent a great source for natural variation studies. Mapping of complex traits in model organisms has, for some time been constrained by low genetic variation of laboratory cultured strains. Although informative, this approach is fairly limiting. Recently, research in many model species has benefited from the creation of multi-parental crosses derived from wild-caught strains. Studying natural genetic variation using this approach allows for a better understanding of gene function as allelic interactions in divergent genetic backgrounds play important roles in determining complex traits. Such an approach was notably missing in *Caenorhabditis elegans* research. To remedy this, a new 4-parental recombination inbred line (RIL) panel that is representative of genotypically distinct groups of *C. elegans* isolates and distinct from the canonical N2 strain has been created. In this thesis I have used *C. elegans*, that has a short lifespan, high fecundity and wide array of genetic and genomic resources, to investigate variation in lifespan, its related traits and in the response to dietary restriction (DR). My specific aims were to further characterise a number of previously isolated lifespan QTLs, and to analyse lifespan and stress resistance in a new panel of 4-parent RILs. This work has discovered that the effect of DR on lifespan in *C. elegans* varies between genotypes and that such differences are seen in introgression lines (ILs), RILs and in wild isolates. A wide review of the literature on DR shows support for the view that genotype-specific effects on lifespan are widespread and that for some genotypes DR can be deleterious. I have also discovered that the newly created 4-parental panel of RILs contains significant, ecologically relevant, variation in lifespan and in stress resistance, that lifespan and stress resistance; are not correlated in these lines, and that this can be used to identify quantitative trait loci (QTLs) controlling this variation. Importantly,

some of these QTLs cannot be explained by known lifespan regulating genes. Furthermore, the analyses revealed that the cold stress resistance in *C. elegans* is related to the control of translation, that the major QTLs detected in the RILs cannot be a consequence of genes known to be involved in cold stress resistance and that one may be a consequence of variation in *eftu-2* a part of the translation machinery. These results highlight the importance of exploring various genetic backgrounds in quantitative genetics and present a wider picture of the genetic interactions that are likely to be happening in the wild.

CHAPTER ONE: Introduction

1.1 Ageing and lifespan in human populations

There has been a steady growth in life expectancy over the past century. This growth means that more people live, on average, longer that their parents. This global rise in average lifespan seen in the human population in the past 50 years is partly a result of major improvements in public health and medicine and older people are making up an increasing share of the total population. However, the desire for prolonged lifespan brings a new dilemma, as extended lifespan does not always equate to an extension in health span. This is a situation never faced before by humanity and despite great improvements in medical research and health care, the aging population remains profoundly vulnerable to both infectious and non-infectious disease. It is therefore important to seek answers to very serious questions such as will the ever growing ageing population possess long term good health, and/or is there a reasonable chance to reduce severe disability from common diseases and health conditions?

Ageing research therefore aims not just to increase lifespan, but also to understand the biology of ageing in order to improve healthspan and postpone senescence and its related diseases. Senescence is the process of deterioration with age. In developed nations, about 85% of babies born this year will live to at least to their 65th birthday (Human Mortality Database, 2016). Between 2015 and 2050, the proportion of the world's population over 60 years will almost double from 12% to 22% (WHO, 2016). The price for living to this older age is usually the opportunity to witness the ageing and decline of human bodies and at the end the fatal and disablitating diseases that are often paired with prolonged senescence (Olshansky *et al*., 2016). The current trend of life extension, which is linked to age-related diseases, therefore represents the biggest challenge to health care systems world-wide (WHO, 2016). A longer life

brings with it opportunities, not only for older people and their families, but also for societies as a whole. For example, the longer the people live, the more chance of passing on their knowledge to the next generations. There is, however, little evidence to suggest that older people today are experiencing their later years in better health than their parents. While rates of age-related severe disability have declined in high-income countries over the past 30 years, there has been no significant change in mild to moderate disability over the same period (WHO, 2016). This creates a need for systematic research into ageing in both human and non-human models that allows for deeper understanding of healthy ageing.

At the biological level, ageing results from the impact of the accumulation of a wide variety of molecular and cellular damage over time. One definition of ageing is: '*Degenerative changes that occur in an organism over time with cells declining in their capacity to fight pathogens and disease in comparison to those of young organisms, thus, making older organisms more vulnerable'* (Toussaint *et al*., 1992).

Ageing is the major risk factor for so called age-related diseases such as cardiovascular disease, cancer and neurodegeneration (Hoeijmakers, 2009). In mammals, ageing is accompanied by a progressive atrophy of tissues and organs and stochastic damage accumulation to biological macromolecules such as DNA, RNA, proteins and lipids (Olshansky *et al*., 2016). One of the dominant theories about the mechanisms of ageing is that molecular damage that accumulates over time accounts for the systematic decline in older individuals (Kirkwood, 2005). Damage to DNA can be either exogenous (diet, radiation, environment) or endogenous (reactive oxygen species, chemical instability and errors during DNA repairs) (Hoeijmakers, 2001). Damaged DNA can cause genome instability and can produce signalling cascades that can lead to cell death (Rodier *et al.,* 2009) as well as mutations that can be oncogenic (Cha and Yim, 2013). As

an organism ages its cells also decline in their capacity to fight pathogens and disease in comparison to young organism. For example, there is an increase in apoptosis in the tissue of old animals (Shen and Tower, 2009) that therefore makes older organisms more vulnerable (Puca *et al.,* 2001).

The rate at which various organisms age differs greatly across species, an observation that indicates that the mechanism of ageing is governed by underlying genetic differences (Anson *et al*., 2003). Lately, a view has emerged that biological ageing is malleable and when ageing in model organisms is delayed so are the associated age-related diseases and conditions (as reviewed in Olshansky *et al*., 2016). Research based evidence shows that ageing can be delayed in model organisms by behavioural manipulation (dietary restriction) (McCay *et al.* 1935), genetic manipulation (Kenyon, 2010) and to some extent by pharmacological means such as metformin (Barzilai *et al*. 2016), rapamycin (Leontieva *et al*., 2014) and acarbose (Harrison *et al*., 2014). Different methods of lifespan extension can be assigned to mostly independent genetic mechanisms. These mechanisms seem to extend lifespan by both independent and overlapping genetic pathways (Greer and Brunet, 2009).

1.2 Stress and dietary restriction

Stress and lifespan are closely related, with many mutations that alter lifespan also affecting the stress-response and nutrient sensing. For example, the mouse $p66^{shc}$ gene (which controls oxidative stress response) has been shown to induce stress resistance and prolong lifespan (Migliaccio *et al*., 1999). Experiments on stress factors can therefore provide insight into the mechanisms of ageing (Lin *et al*., 1998). Mild stress, also called hormesis can be beneficial for an organism. Hormesis stands for a dose response where low exposure to toxins or other

stressors have an overall favourable effect on a given organism (Calabrese *et al*., 2002). Many animal studies have demonstrated that mild dietary stress can be beneficial to model organisms. In one such study, pathological lesions in rodents due to old age in mice were significantly reduced by a dietary restriction (DR) regimen (Ikeno *et al*., 2006). Moreover, in mice, multiple fasting cycles modulate haematopoietic stem cell protection, self-renewal and regeneration (Cheng *et al*., 2014; Fontana and Partridge, 2015). DR is a regime of decreased food intake and can be defined as low caloric intake without malnutrition (Gredilla and Barja, 2005). It has been shown to markedly and reproducibly extend lifespan in a variety of species (Fontana *et al*., 2010) and appears to be one of the very few near-universal means of lifespan extension in animals. DR is also able to delay age-related diseases such as diabetes, dementia and cardiovascular diseases in a number of model systems (Herndon *et al*., 2002; Mair and Dillin, 2008; Kaufman *et al*., 2010). Dietary restriction is, to this day, the only natural intervention known to reliably extend lifespan in model organisms. For example, DR started in young Rhesus monkeys greatly improves metabolic health and reduces the risk of developing of cardiovascular diseases, obesity and cancer (Colman *et al.,* 2014; Mattison *et al.,* 2012). DR therefore appears to act generally in keeping organisms relatively healthy until older age. DR therefore represents a robust way to improve both lifespan and healthspan.

Early in the twentieth century, the first recorded DR experiments were carried out to observe how retardation of growth due to malnutrition in rats and trout effected lifespan (Osborne and Mendel, 1915; Osborne *et al.,* 1917 & McCay, 1929). It was McCay who first noticed that growth retarded trout, outlived the control population (McCay, 1929) and his 1935 research on rats is now most commonly cited as the beginning of modern DR studies (McCay *et al.,* 1935). During the past 100 years many different species from wide taxonomic groups from

rotifers (Fanestil and Barrows, 1965), worms (Lee *et al*., 2006), flies (Mair *at al.,* 2005), spiders (Austad, 1989), fish (Terzibasi *et al*., 2009), rats and mice (Turturro *et al*., 1999) to monkeys (Colman *et al*., 2009) have been used in DR research and, to this day, DR is considered the most robust non-genetic life-extending intervention. Historically an important question has been to determine the degree to which studies on ageing on non-human organisms are informative about the mechanisms of human ageing. Although it is not possible to answer this question definitely, there is a large body of work supporting the idea that the basic principles of ageing are broadly conserved across the species as identified in commonly used model organisms (Kenyon et al., 1993; Sutphin and Kaeberlein, 2011).

Beyond the potentiality of DR to affect human lifespan, DR studies on animal models have been extremely useful in defining genes and molecular pathways involved in ageing, some of which appear to be greatly conserved, *e.g.* insulin-like signalling which was originally shown to regulate lifespan in *C. elegans* (Kenyon *et al.,* 1993). Many animal models from wide taxonomic groups have however shown no lifespan extension and even a reduced lifespan under various DR regimens. This raises the possibility that DR may not be as universal a mechanism of lifespan extension as has been suggested. Further, if there are indeed genotype-specific effects of DR, this may explain some cases where conflicting results have been obtained.

There are, some perplexing questions still to be answered about how DR evolved and why it extends lifespan. DR-extended lifespan is mostly, but not always, coupled with hypothesis of reduced rates of reproduction (Partridge *et al*., 2005). One of the earlier evolutionary hypothesis was the re-allocation of the nutrients in DR treated organisms. The bases of this theory was simple, in time of plenty, an organism invests in growth and reproduction, but when resources are scares the nutrients are re-allocated for maintenance and repair (Speakman and

Mitchell, 2011). This idea, when applied especially to invertebrates, appears to be at odds with the boom and bust lifecycles of many of the species such as yeast, worms and flies (Adler and Bonduriansky, 2014). Also, given that most of the animals in nature do not live till old age, saving resources for later and postponing reproduction may not be the best strategy. Furthermore, many species that are capable of surviving longer periods of unfavourable conditions often do so via more extreme strategies such as alternative developmental stages, for example dauer larval stage of *C. elegans* (Hu, 2007). This allows the organism to survive and develop into an adult once the conditions become favourable again.

In addition, long term dietary restriction treatments cause many physiological changes in the organisms. One of these changes, is the reduction in the capacity to respond to environmental challenges. Indeed, when exposed to pathogens, DR restricted animals tend to exhibit reduced immune response with increased susceptibility to viruses, bacterial infections and gut parasites (Kristan, 2008; Speakmen and Mitchel, 2011; Hunt et al., 2012). There is also some evidence that cold tolerance is compromised under DR regimens (Puerta and Abelenda, 1987). As most DR experiments are carried out in laboratory conditions with no predators or competitions for mates, a sedentary lifestyle (He *et al.,* 2012) and stable temperatures, such physiological changes could cause increased mortality in the wild, *i.e.* what is interpreted as a lifespan extending DR effect in the laboratory would shorten lifespan in the wild. For example, Voorhies *et al*. (2005) reported a greatly reduced longevity for *C. elegans* when cultured in soil in comparison to worms reared on NGM plates. These arguments, coupled with the results of many non-model organisms and wild-derived strains in which DR failed to prolong lifespan (reviewed below), are suggestive that response to DR are not due to re-allocation hypothesis.

1.3 Mechanism(s) of DR

There are two principal methods of restricting diet in both vertebrate and invertebrate organisms. In the first method, chronic DR, daily calories are restricted by typically between 20-40% of *ad libitum*. When started at an early age, animals on this regimen are generally smaller in size when compared to the control (Rusli *et al.*, 2015). In the second method, food is withdrawn altogether on non-consecutive days such as alternate day fasting (ADF), intermittent fasting (IF) or for timed periods with *ad libitum* feeding on non-fasting days/times. In rodents, fasting every other day, or twice weekly extends lifespan up to 30 % regardless of both total food intake and weight loss (Mattson *et al*., 2014). Animal models in these trials are of a normal size when compared to control groups (Descamps *et al*., 2005). This physical difference between various treatments is a good indication that various DR methods operate via independent or partially independent pathways (Greer and Brunet, 2009). The hope is that a better understanding of these mechanisms can also provide the means to prevent or delay the onset of age-related diseases in an ever ageing and expanding human population. It is also hoped that understanding the mechanism of DR will allow pharmacological replication of the effect therefore allowing DR extension of lifespan without the requirement for DR.

The mechanisms underlying the anti-ageing effects of DR have long been a major interest to researchers. Initially it was proposed that various DR regimes increased lifespan by retarding growth. This hypothesis was soon eliminated and the search began for new mechanisms. The discovery that certain mutations can extend lifespan without the nutritional intervention by manipulating of the cellular signalling greatly facilitated this search for the genetic mechanism(s) of DR (Kenyon *et al*., 1993; Kenyon, 2010; Fontana *et al*.,2010). The protective effect of DR has been attributed to a reduction in oxidative damage and protection of

mitochondrial structures (Anderson and Weindruch, 2010). It has been reported that DR can reduce mitochondrial proton leakage and increase antioxidant genes responsible for slowing ROS (Sreekumar *et al.,* 2002). DR is also thought to operate, at least in part, by decreasing mTORC1 signalling. Inhibition of the mTOR signalling pathway promotes longevity in a variety of species (as reviewed in Kapahi *et al*., 2010). The mTOR signalling network exists in two complexes, mTORC1 and mTORC2. The insulin/IGF-1-like signalling pathway and associated FOXO transcription factors also play an important role in the signal transduction pathway associated with stress and ageing phenotypes. In *C. elegans* this pathway is central to both growth and metabolism (Murphy, 2006; Tullet *et al.,* 2008).

1.4 DR not working in model organisms

Despite the plethora of research indicating that DR can slow ageing, there are numerous examples where DR either fails to extend the lifespan of an organism or can even shorten it. For example, DR in 166 strains of the yeast *Saccharomyces cerevisiae* showed changes in the median lifespan ranging from a 79% reduction to a 103% increase in replicative lifespan (RLS) (Schleit *et al*., 2013). In strains with reduced RLS, genes from the pathways regulating cellular and organellar oxidative stress, ion homeostasis and pH were significantly enriched (Schleit *et al.,* 2013). In contrast, those strains showing the most positive response to DR showed upregulation of many genes involved in mitochondrial functions. This is not a surprise as mitochondria are known to have a central role in ageing-related diseases and mitochondrial dysfunction is known to lead to increased oxidative stress (Lin and Beal, 2006). The data in this study is consistent with an analysis of the mean lifespan of virgin males and females from 41 mouse recombinant inbred lines (RILs, Liao *et al*., 2010). This study revealed great variation in

lifespan between RILs with a less than 40% reduction in calories (the level of restriction most common in DR studies in rodents as any regime above 40% is considered a starvation), with two of the RILs (5%) showing a significant extension in male lifespan under DR conditions and eight RILs (21%) showing a DR-induced increase in female lifespan (Liao *et al*., 2010). Even more, DR appeared to greatly decrease the mean lifespan for males and females (27% and 26% of the RILs respectively). One possible explanations for this rather surprising outcome is the small sample size and housing conditions, as commented by Mattson (2010). The explanation for these observations it that the most aggressive animal (males are more aggressive than females) would consume more food, leaving the remaining mice with a more severe form of DR. This potentially explains the greater number of RILs showing an increase in female lifespan than male lifespan, with the male DR treatment being equivalent to one well-fed animal and the remainder being under starvation conditions. In addition, the sample number in each group is very small (n=5) and the study did not determine the cause of death, which is common practise in most DR studies.

There are many factors that play important roles in DR studies such as the choice of the strains used, fitness and age of the animals at the beginning of the experiment, housing and treatment of the animals, variation of the DR regimens and, last but not least, genetic variation. One of the biggest issues within DR studies is the choice of strain. For example, in mice research, the most popular strain is the C57Bl/6 (C57 Black). This most widely used strain is used for its robustness and easy breeding. However, animals of this strain are over sensitive to pain and cold and partly resistant to analgesic medications (Mogil *et al.,* 1999). C57Bl/6 mice also have a relatively low bone density when compared to other strains, are prone to developing hearing problems and are susceptible to diet-induced obesity (the Jacksons Laboratory, 2015). This

combination of traits suggests inbreeding depression, with the fixation of many deleterious alleles within the strain and the susceptibility to diet-induced obesity further suggests a potential issue with insulin-like signalling. In one of the DR experiments using C57Bl/6 strain, the mean lifespan of juvenile male mice was significantly decreased, but the lifespan of the mice once they reached adulthood was not affected by the changes in their diet (Silberberg *et al*., 1961).

The poor choice of strains in DR studies is also prominent in *C. elegans* studies. Most studies have used one canonical strain, Bristol (N2) as a reference genotype. The N2 strain is generally referred to as the 'wild-type' isolate. This classification is, however, questionable as the N2 strain has been maintained in continuous culture for about 13 years prior to freezing and thus subjected to very different condition to that of in the wild (Chen *et al*., 2006; Petersen *et al*., 2015; Sterken *et al*., 2015). The N2 'wild-type' has therefore extensively adapted to laboratory conditions (Sterken *et al*., 2015) and the genes governing the response to DR could have been affected by this. One study that has used freshly derived wild isolates of *C. elegans* and *C. remanei* showed that DR treatment extended the lifespan in *C. elegans* populations with various effects and, in *C. remanei*, robustly extended mean lifespan in one line whereas in the other strain the maximum, but not mean lifespan, was extended (Sutphin and Kaeberlein 2008). Both nematode species showed substantial variation in lifespan and response to DR. Outcomes such as this may be due to different characteristics between the two species. *C. elegans* hermaphrodites were not allowed to mate with males whilst *C. remanei* has males and females and, although lifespan data was only collected from females, the worms were allowed to mate.

Similar results have also been seen in a recent *C. elegans* study that focused on natural variation in lifespan and response to dietary restriction by peptone deprivation in wild-derived isolates and five introgression lines (ILs) of N2/CB4856 in this thesis (Chapter 2/Stastna *et al*, 2015). Each of these ILs contains an introgressed portion of the CB4856 genome in an N2 genetic background and had previously been shown to have a lower lifespan than N2 (Doroszuk *et al*., 2009). This study discovered 3 NILs in which DR failed to prolong lifespan and showed increased maternal hatching (*C. elegans* response to poor life conditions such as food deprivation). A great variation in lifespan and in the effect of DR was also observed in wild isolates from various locations in France and the Netherlands. The genetic and gene expression differences between these wild isolates are characterized by gene-environment signatures (Volkers *et al*., 2013) and it is therefore reasonable to expect that different isolates will display a differential response to DR treatment. Seven out of twenty-two wild isolates were shorter lived in comparison to control conditions and an additional three isolates showed negligible effects of DR on mean lifespan. Together with the results of the NIL analysis, this shows that DR is not a universal method of lifespan extension within genotypes obtained from specific crosses as well as genotypes freshly taken from their natural habitat.

The importance of using wild isolates in DR study is slowly emerging. One such study has looked into dietary restriction in freshly caught wild mice (Harper *et al*., 2006). The survival in mice under 40% DR did not significantly differ between the two cohorts. Control animals lived on average 888.5 days and DR animals 870.6 days. As seen in previous DR experiments with wild-derived animals, many animals died early in the experiment (e.g. Terzibasi *et al*., 2009), however, the longest lived animals (8.1%) were from the DR group. Even if the effects of DR were not projected into the prolongation of lifespan, the necropsy results revealed a dramatic

antitumor effect of the DR treatment with 60% of control animals having tumours compared to only 12% of dietary restricted ones. This result seems consistent with work on the antitumor effect of DR due to elevation of circulatory corticosterone (Birt *et al*., 2004).

As mentioned previously, Terzibasi *et al*. (2009) also looked into comparing lab adapted animals to wild-caught ones. The results showed an increase in the maximum lifespan in the lab inbred GRZ strain, whereas in the wild-derived MZM-04/10P strain, DR resulted in early mortality between week 4 and week 15 of treatment, eliminating 37% of the experimental animals. As a result, the mean lifespan was not increased, but the maximum longevity was extended by DR (data not significant). Also in both lines, DR prevented the expression of the age marker lipofuscin.

One of the possible explanations for the difference between wild-derived strains and lab adapted strains is that there are hidden fitness trade-offs. Such trade-offs could be associated with life history traits that have a strong effect on natural populations, but not in laboratory populations. One such trade-off could be the immune system as it is costly to maintain and may be included in the complex of trade-offs associated with the evolution of life history traits (Reznick and Ghalambor, 2005). There are similar conclusions in *C. elegans* following the comparisons of lifespans in laboratory strains *age-1* (hx546) versus wild type (Walker *et al.*, 2000) and mice, where two of the wild derived mice lived longer than the lab inbred one (Miller *et al*., 2002).

1.5 DR not working in non-model organisms

Most papers on DR present a plethora of very successful examples of the various regiments from many model organisms. There are however many studies on non-model organisms where

DR fails to increase lifespan. For instance, in rotifers DR increased lifespan in most of the species tested, but decreased mean lifespan in three species (Kirk, 2001). Similarly, in Mediterranean fruit flies (Medfly) no increase in longevity was observed at any level of diet restriction (100%-30% of *at libitum*) (Carey *et al*., 2002). This study also reported that each sex responded differently to treatments and that reproduction changes the impact of DR on longevity (Carey *et al*., 2002). One of the possible explanations is that Medfly differs from *Drosophila* in terms of their physiological response to nutritional ecology. Another example from the *Diptera* is the housefly, where DR progressively shortened the lifespan of male house flies that were feeding on sucrose, with increasing severity of DR was reflected in a progressively shortened lifespan (Cooper *et al*., 2004). These results seem rather inconsistent with studies on the closely related fruit fly *Drosophila melanogaster* (see for example Min *et al.,* 2007). Here DR was achieved by altering the concentrations of glucose and yeast in the medium. Lifespan was also not prolonged by various diets in Lepidoptera (Beck, 2007; Saastamoinen *et al*., 2010; Niitepold *et al*., 2014) and in some of the studies of DR on spiders. For example, DR failed to increase longevity of male Australian redback spiders (Kasumovic *et al*., 2009). In contrast, Austad (1989) observed a strong inverse relationship between the lifespans of the lab cultured female doily spiders, *Frontinella pyramitela*, and the feeding regime for spiders fed 1, 3 or 5 *D. melanogaster* per week (mean lifespans of 81.3, 63.9 and 42.3 days, respectively). This study did however also observe a strikingly short lifespan (mean of 8.1 days) for *F. pyramitela* females in the wild. Are these results possible proof that an increase in lifespan by DR is in fact an artefact of the lab culture, where animals are most likely to be overfed and do not have to face same challenges such as predators, temperature fluctuation and viruses for example? Or is the reason that the scientists were unable to see any lifespan increase under DR regiments a mere reflection of natural populations and the events that are happening in the wild? As the partial

loss of the plasticity reflected in added lifespan in the lab is just an energy that must be spent in order to survive in the wild.

1.6 Timing of food restriction

The abundance of food for humans and domesticated animals has only been a trend of the very recent past. It is unlikely that food used to be in constant supply and therefore most species are likely to have evolved under conditions in which periods of fast/starvation were likely to occur. It is therefore likely that natural selection will have optimised fitness for such conditions. Food, when found, would be consumed immediately leaving periods of hunger as observed in DR mice fed only once a day where food gets consumed very rapidly leaving ~22-23 hours between feeds (Swindell *et al*., 2012). Such mild dietary stress seems very similar to that of intermittent fasting (IF) (consuming all/or most of the daily calories in short period of time). The difference in feeding regimens could be a possible explanation as to how two recent DR studies in rhesus macaques (*Macaca mulatta*) came to different conclusions. Although both studies generally came to observe that DR positively regulates metabolic markers such as glucose, there are different outcomes when it came to lifespan. In Colman *et al*. (2009) the effect of DR is a clear reduction in mortality. At the time point reported, 80% of the animals from the DR group survived in comparison to 50% of the control group. Also, out of the control group, 37% of animals died due to age-related diseases compared to the 13% from the dietary restricted group (results confirmed by a complete necropsy, Colman *et al.,* 2009). Results of the study by Mattison *et al.* (2012) contrasts with this, finding that DR did not improve survival outcomes. The DR treatment was, however, beneficial on several measures of metabolic health such as triglycerides, cholesterol and glucose levels. In this study, DR was implemented in

monkeys of various ages (young, middle and older ages). In another study on mature animals, Barrows and Roeder (1965) reported that DR restriction through adulthood in the Sprague-Dawley strain of rats failed to increase lifespan in these animals. The two groups of adult animals (12 and 18 months old) were subjected to a DR of 50% to that of *ad libitum*. The mean lifespan of either group of animals was not greater than that of the control (*ad libitum*). The severity of the restriction imposed was greater than that observed in other studies, as most of the DR studies use between a 10-40% reduction of food to *ad libitum*. In similar experiments, two mice strains (C57BL/6 and DBA/2) and an F1 hybrid (B6D2F1) were tested (Forster *et al*., 2003). Here, DR prolonged the lifespan of one of the strains C57BL/6 and the F1 hybrid B6D2F1, whereas in mice of strain DBA/2, DR resulted in a slightly shortened lifespan. This suggests that genetic factors either modulate resistance to the effect of DR or reversely affect tolerance to the detrimental effects of DR. This highlights the importance of genetic background in DR studies, or rather lack of it.

1.7 Genetic background

There are several factors that play a role in the choice of strains in DR studies. First and foremost, the strain selection is influenced by available genetic information. As mentioned previously most of the DR studies in mice are carried out in C57Bl/6 strain. In most of these studies, DR treatment results in lifespan extension (Anson *et al*., 2003; Rusli *et al*., 2015). This approach, however, does create a rather unrealistic scenario, where a strain that is genetically susceptible to obesity and other maladies (Mogil *et al.*, 1999) is used as a model for human ageing. In studies in which more than one strain was used, the results are somewhat different. For example in Forster *et al*. (2003), DR prolonged lifespan in C57BL/6 and a hybrid of this strain,

but resulted in lifespan shortening in DBA/2 mice. Another study used four genotypes of mice and three genotypes of rats to identify and validate a panel of biomarkers for ageing in rodents to provide the basis for development of biomarkers for ageing in humans (Turturro *et al.*, 1999). Here, although DR prolonged lifespan in all animals in general, the effect within inbred laboratory mice varied greatly between the sexes and was hugely depended on genetic background. This suggests that genetic factors either modulate resistance to the effect of DR or affect tolerance to the detrimental effects of DR. The second outcome of this study is the finding that DR administered in advanced age did not benefit the rodents and, depending on the genotype, had deleterious effects (Turturro *et al.*, 1999). This suggests that DR in advanced age may be harmful to animals and potentially to humans.

Another important issue in various DR studies is the use of mutants. The reason for this is because mutations are usually analysed in only a single genetic background (Kammenga *et al.,* 2008). This could be a cause for concern because studying of genetic function in one genotype does not allow for understanding natural genetic variation and allelic interactions in different genetic background plays an important role in determining genetic basis of complex traits (Kammenga *et al.,* 2008). Furthermore, induced mutants are normally outcompeted by wild isolates when subjected to more natural conditions even in cases where there is no apparent fitness cost under benign conditions. This is most strikingly illustrated in comparisons of long-lived *C. elegans* mutants, which are outcompeted by the wild type only under conditions of cyclic starvation (Walker *et al.*, 2000). This also suggests that when conditions are not environmentally challenging, genetic lifespan extension can be uncoupled from other life history traits.

When animals are kept under laboratory conditions with good availability of food, no predators, nor competition for mates, there is the possibility that this can lead to a gradual loss of the natural alleles that influence ageing. One of such example is in mice, Miler *et al*. (2002) studied three lines that were derived from the wild population. Lines used were Id, Ma and Po, these were compared with the genetically heterogeneous stock DC. The results were striking, the laboratory line, DC was much faster to reach sexual maturity and was also the shortest lived strain. The wild-derived mice all outlived the laboratory mouse with one of the strains dying at the maximum age of 1,450 days- the longest for fully fed non-genetically mutant mice (Miler et al., 2002). The reason for such variation is unknown, however, it is most likely to be the effect of most of the studies are focused on genotypes exhibiting great effects when under DR.

1.8 Summary of DR

Nearly a century of laboratory research has yielded a plethora of experimental data on various DR methods. These extensive studies have helped in understanding that ageing is subjected to genetic regulation and is thus, at least partially, malleable. Also, the outcomes from various studies have linked metabolism to ageing and uncovered the importance of cellular signalling. Although DR tends to increase lifespan in the protective and benign environment of a laboratory, much research still needs to be carried out in order to understand the ecology and evolutionary implication of DR. Overall, DR still seems to be a robust non-genetic intervention capable of postponing not only senescence but also age-related diseases in most model organisms. However, there is sufficient evidence to suggest that genetic background and, in particular, various types of inbreeding and lab adaptation can result in differing responses.

Given this, future studies on DR could be greatly improved by the use of freshly-derived wild isolates alongside the various routinely used laboratory strains. Also the research would greatly benefit from determining the natural nutritional conditions for individual species in wild populations and incorporating this into the laboratory environment. A central message from much of this work is the need to consider that the complex development of the senescent phenotype is the product of genes, environment, and intrinsic stochastic factors (chance). Also testing on wider range of species should be adopted to allow for better understanding of the various DR effects on an organism. Both sexes, especially in rodent studies, should be used as well as allowing for more realistic environments which includes breeding and more varied diets.

1.9 Ageing and lifespan in model organisms

For centuries, ageing and reversing the ageing processes have been of great interest to humans. The paradigm is that every living organism ages, however, the speed of ageing is different among various species as well as the variation within species. New ideas are emerging among scientists to shift this paradigm and postpone or stop ageing altogether. As a definition,' *Ageing is an intrinsic as well as a universal process that happens in all individuals of all species and it is progressive and deleterious to all living organisms'* (Harman, 1955). Over the years, many theories of ageing have been postulated amongst scientists with Mutation accumulation, Hayflick limit (telomere shortening), Waste accumulation theory, Free radicals' theory, Antagonistic pleiotropy and Disposable some theory, being a few of the major ones. For example, the disposable soma theory favoured by Thomas Kirkwood (1977) states that the organism has only limited amount of energy that has to be divided between reproductive

activities and somatic maintenance. Although considered to be a great idea at the time, this theory failed to explain why females use more resources for reproduction and still outlive males (Barford *et al*., 2006). In most wild animal populations, the individuals do not reach the senescent 'old age' stage but rather die young due to extrinsic mortality risks (Kawasaki *et al*., 2008). This selective effect underpins the antagonistic pleiotropy theory. Here, alleles that increase fitness early in life are beneficial, and hence selected for, even if they are disadvantageous at old age (Rose, 1982).

Another class of theories relies on the accumulation of either waste products or of damage and on the subsequent effects of this on the organism. The accumulative waste theory proposes that organisms age due to oxidation and its by-products, causing dysfunction, toxicity and eventually cell death (Harman, 1955). The wear and tear theory is related to this and is based on the view that ageing is caused by mutation accumulation resulting from radiation, UV light, toxins and free radicals such as reactive oxygen species (ROS) and reactive nitrogen species (RNS; Afanasev, 2010). This results in progressive and age-related decline in fitness. Another related theory is the free radical theory introduced by Harman nearly fifty years ago, which remains one of the most popular and best known theories of ageing. It proposes that molecular components are damaged by free radicals generated during normal metabolism. This damage can lead to decreases in production of ATP, oxidative stress damage to proteins and eventually cell senescence (Lopez-Otin *et al*., 2013). However, not all experimental findings support this theory as, for example, it cannot explain how physical exercise can be beneficial if it increases free radicals within the organism (Afanasef, 2010) or why increasing oxidative stress in *C. elegans* does not necessarily lead to a shortening of lifespan (Raamsdong & Hekimi, 2009).

Beyond the evolutionary explanations for lifespan, it is clear that stochastic processes greatly influence lifespan as there is great variation in observed lifespan between similar, and even identical, individuals (Herndon *et al.,* 2002). For these reasons, prediction of the lifespan of an organism by chronological age is not always easy, a situation that creates a need for biomarkers. Biomarkers for ageing are predictors of the functional capacity of a given organism that also offer validating power to experiments on ageing. One such recognised biomarker for ageing is the waste product lipofuscin. The rate of lipofuscin's accumulation in post mitotic cells such as neurons, skeletal muscle fibres and cardiac myocytes is known to increase with age and is now recognised as one of the hallmarks of ageing (Brunk and Terman, 2002).

1.10 Model organisms

The mechanisms that underlie ageing have, thanks to progress in genetic methods, been now extensively studied in many systems. Animal models provide a powerful platform to study basic biological functions, with many mechanisms and processes being found to be highly conserved across species, meaning that model organisms allow for a close approximation to a humans' response. One of the major challenges in studying ageing is to define the boundaries of normal change and those of pathological conditions. The boundaries that may be difficult to observe in humans are potentially more visible in model organisms and the use of laboratory animals enables various pathologies to be studied in a systematic way (Gallagher and Rapp, 1997).

The most extensively studied model systems for work on ageing are yeast (Bell, 2010), flies (Mair *et al.,* 2005), worms (Bishop and Guarente, 2007), the vertebrate model species are mice (Reed *et al.,* 1996), rats (Stein *et al*., 2012) and rhesus monkeys (McKiernan *et al*., 2012; Fontana *et al*., 2010). Model species have long been used in longevity research and the

vertebrate model species are especially of high importance. For the past 100 years, these models were two rodent species, rats and mice (Osborne and Mendel, 1915; Robertson and Ray, 1920). In the 1930's, two reports from the McCay laboratory were responsible for directly linking ageing to metabolism (McCay *et al.*, 1935 and McCay *et al.,* 1939). These set the precedence for DR experiments which later became the gold standard for DR studies in other model organisms. Research has since moved on to study the effects of DR on longevity and nowadays there are a plethora of methods for lowering caloric intake. Many studies on mice (Rusli *et al.,* 2015), rats (Stein *et al*., 2012) and rhesus monkeys (McKiernan *et al*., 2012) now postulate that DR may postpone or reverse age-related diseases in mammals. Such studies into ageing and age-related diseases are extremely important in areas where many experiments are not possible such as in humans (Partridge, 2009). In humans, DR results in improvement of several markers of health (Fontana and Partridge, 2015).

Unicellular and invertebrate models also provide excellent model systems for research into ageing due to their short lifespan and prolific reproduction capacity. Numerous studies have been carried out using vast arrays of experiments on stress, postponed senescence and fecundity in yeast (Bell, 2010), flies (Mair *et al*., 2005), and worms (Bishop and Guarente, 2007). At present, however, not much is understood about the underlying genetic control of the effect of dietary restriction on lifespan, nor the effect of prolonged DR on the human body. The budding yeast *Saccharomyces cerevisiae* is one of the most important models used for ageing. While some aspects of ageing in yeast are organism specific, there are many important features that have been highly conserved across species (Kaberlein, 2010). Ageing in yeast is measured by monitoring the number of daughter cells generated by an individual's mother cells. This process is called replicative life span (RLS) or by observing for non-dividing cells, chronological

life span (CLS; Mortimer and Johnston, 1959). In *S. cerevisiae*, glucose, amino acids and other nutrients can activate pathways including Ras, adenylate cyclase (AC), protein kinase Aber (PKA) and the pathway including the mechanistic target of rapamycin (mTOR; Fontana *et al*., 2013). Rapamycin is an immunosuppressive macroline serine threonine kinase Sch9, first discovered in bacteria on Easter Island (Heitman *et al.,* 1991) and was identified genetically by mutation in yeast (Fabrizio *et al*., 2001). Deletion or inactivation of various components of these pathways delays both replicative and chronological lifespans in yeast (Lin *et al*., 2000) as well as delay aging in worms, flies and mice (Kaeberlein *et al*., 2005).

The round worm *Caenorhabditis elegans* is a facultative hermaphrodite. This multicellular animal model provides a plethora of advantages for ageing studies. *C. elegans* has a relatively short life-cycle – reaching adulthood in just 3.5 days at 20^0C. After hatching, the worm moults through 4 larval stages (L1 – L4) and once it reaches adulthood, the average lifespan is approximately 18 days under lab conditions (Byerly *et al.,* 1976). In *C. elegans,* insulin/IGF-1-like signalling pathway (ISL) is essential for both growth and body size (Oldham and Hafen, 2003). Cell growth in response to nutrients is also controlled by the highly conserved mTOR (mechanical target of rapamycin, Schmelzle and Hall, 2000) and S6 kinase (Panowski and Dillin, 2009). The relationship between ageing and reproduction is particularly obvious in *C. elegans*. The worm's germline is integral to nutrient signalling and communicates with other issues to modulate ageing (Kenyon, 2010). Loss of the germline activates the insulin/IGF-1 pathway and result in lifespan extension by 40-60% (Hsin and Kenyon, 1999). If the germline precursor cells are removed in flies, then this also leads to extended lifespan (Flatt, 2008).

The fruit fly *Drosophila melanogaster* is another major organism used in ageing research. Studies using flies date back to 1916 when Loeb and Northrop linked temperature changes to

lifespan (Loeb and Northrop, 1916). The fly has three distinguishable growth stages that occur over 10 days; embryo, larva and pupae followed by adulthood of 2-3 months at 25^0C (Caravaca and Lei, 2016). Unlike worms and yeast, fruit flies cannot be cryopreserved to provide a 'fossil' record, an issue for certain types of analyses and experiments. Nutrient signalling and food availability in *Drosophila* have been shown to be critical factors in regulating growth, size and lifespan (Oldham and Hafen, 2003). The main pathways include InR/PI3K, TOR, insulin/insulin-like growth factor signalling (IIS) and S6 kinase (Kaeberlein *et al*., 2005). It was found in *Drosophila* that reduced IIS can prolong the lifespan that established the evolutionary role of this pathway in ageing (Piper *et al*., 2008).

The mammalian model mouse, *Mus musculus* is without a doubt the most important animal model for ageing due to its proximity to human biology. Mice have a relatively short lifespans, although these can vary greatly among strains. The most commonly used strain for aging has been the C57BL/6, the 'black 6' used for its robustness and easy breeding (Mogil *et al.*, 1999). Also, it was this strain whose genome was published in 2002 (Chinwalla *et al.,* 2002). Nutrient signalling is crucial in healthy ageing, for example, it has been shown that the reduction in mTOR activity extends lifespan in rodents (Johnson *et al.,* 2014).

Studies from these model organisms have identified several evolutionary conserved pathways and genes that share similarity in modulating longevity across broad taxa. The main longevity pathways include: mTOR, an essential component of two distinct complexes, mTORC1 and mTORC2 that regulate a variety of cellular activities (Johnson *et al.,* 2014); the Sirtuins, a family of NAD^+ dependent enzymes; adenosine monophosphate-activated kinase (AMPK); insulin-like growth factor1 (IGF-1) signalling; and the mitochondrial stress and antioxidant pathways (Olshansky *et al*., 2016). There seems to be a general principle in which conserved modifiers of

longevity tend to regulate the relationship between growth and reproduction via nutrient status. However, many of these mechanisms are still poorly understood which creates a need for more research to gain a better understanding of the connections between these biochemical pathways.

1.11 *Caenorhabditis elegans* as a model organism

Why study worms? The nematode *Caenorhabditis elegans* is one of the leading model systems in biological research. Due to their gene homology, *C. elegans* provides one of the fastest, cheapest and most efficient ways to learn about ourselves (Riddle, 1997; Kammenga *et al*., 2008). Knowledge from one small organism, even as simple as a worm, can provide us with a great power when connected to findings from other organisms. Sydney Brenner first introduced *C. elegans* as a model organism for developmental genetics in 1963. It was also Brenner who proposed using *C. elegans* in order to identify every cell in the worm and to trace the cell lineage. Since then *C. elegans* has become established as a standard experimental system for the investigation of a great variety of biological processes: aging, sex determination, cell lineage, cell death, myogenesis and neural development and specification to name a few (Hodgkin and Doniach, 1997; Eckmann *et al*., 2002; Herndon *et al*., 2002).

Reproduction occurs by either self-fertilization in hermaphrodites (approx. 300 per single hermaphrodite) or by mating with males (up to 1,000 progeny) which makes *C. elegans* a great model for genetic analysis. The hermaphrodites have two XX chromosomes and males have one X chromosome, therefore XO. The mode of reproduction in nature is mostly by hermaphrodite selfing, with males occurring at about <1% of total worm number in the progeny of self-progeny of hermaphrodites (Barriere and Felix, 2005). In nature, there is low

frequency of males to hermaphrodites; probably due to the fact that reproduction by hermaphrodite selfing is more efficient for the production of oocytes (Hodgkin and Barnes, 1991). One of the main advantages of using *C. elegans* as a model organism for genetic studies is that it does not suffer from reduced biological fitness in given population as a result of inbreeding (called inbreeding depression or hybrid vigour; Dolgin *et al.,* 2007). In 1998, this free-living and globally dispersed worm was the first multicellular animal to have the whole genome fully sequenced (Sequencing Consortium, 1998). The large number of genome sequences now available and the well-curated genome in combination with the extensive knowledge of cellular and neurological processes now make *C. elegans* an excellent model for quantitative systems biology (Gaertner and Phillips, 2011).

1.11.1 History of *C. elegans*

French zoologist Emile Maupas, who first described *C. elegans* in 1900 (Maupas, 1900), thought the nematode was primarily a soil dwelling organism, and this classification has been frequently repeated in numerous papers (Donkin and Dusenbery, 1993; Walker *et al*., 2000; Liu *et al.,* 2010). However, more recent work has determined that *C. elegans* is a colonizer of various microbe rich habitats such as compost-like heaps and rotting plant materials and have been reliably found in different types of rotting plant material such as fruit, stems and flowers and in association with other invertebrates (Felix and Braendle, 2010; Frezal and Felix, 2015).

Since its introduction into the research community by Sydney Brenner in the 1960's and the first publication of genetic screens in 1974 (Brenner, 1974), the N2 Bristol strain has been widely used in laboratories all over the world and has become the canonical wild type strain. N2 was derived from the Bristol culture that Sydney Brenner had obtained from Professor

Ellsworth Dougherty in 1964, as a liquid axenic culture (Brenner, 1974). The ancestor of Bristol (N2) was originally collected by L.N. Staniland from mushroom compost near Bristol and is descended of the progeny of a single hermaphrodite (Nicholas *et al*., 1959). N2 spread from the Laboratory of Molecular Biology in Cambridge into laboratories all over the world. However, N2 was maintained in continuous lab culture for about 13 years prior to cryopreservation (cooling of the whole organism to very low temperatures), which are conditions unlike those found in the wild. This has led to the accumulation and selection of random mutations and extensive laboratory adaptations (reviewed in Sterken *et al*., 2015). For example, comparing N2 to most of the available natural isolates, uncovered differences between N2 and other wild types in copulatory plug formation in males (Hodgkin and Doniach, 1997). N2 alleles of *npr-1* and *glb-5*, which co-operate to affect behavioural responses to O_2 and CO_2, also originated as adaptations to laboratory conditions (McGrath *et al*., 2009). Other research reported variation in *npr-1* which caused reduced numbers of dauer larvae formation in N2 (Green *et al*., 2013). Duveau and Félix (2012) have identified *nath-10,* a gene affecting vulval development in N2 but not in other wild type strains, and worms with this allele express a strong competitive advantage under laboratory conditions. For review of domestication of N2 in lab condition see Sterken *et al*. (2015). This extensive lab adaptation is not surprising given that N2 has been kept in lab conditions for many generations and was possibly exposed to numerous bottlenecks (big losses of genetic variation; Felix and Braendle, 2010). Additionally, differences in culturing techniques used by laboratories across the world have resulted in further differences in phenotypes within this wild type strain (Vergara *et al.,* 2009, Gems and Riddle, 2000), *i.e.* N2 from one laboratory may be genetically and phenotypically different from N2 from another laboratory. Given that nearly all research in *C. elegans* biology has been performed in N2 and on mutants produced in an N2 background, there is clearly a

need for research that can explore the full potential of the natural genetic variation in wild-derived populations.

It is also worth mentioning another strain with a great historical importance, the Bergerac (BO) strain. This wild isolate was obtained from the soil in France by Victor Nigon and Dougherty (1949). The BO strain exhibits high rates of spontaneous mutation due to a loss of repression of the transposon Tc1 and the subsequent multiplication and movement of that element (Riddle *et al*., 1996). The BO strain has been crossed repeatedly with the Bristol strain for several reasons; one was to create 'mutator' strains which were later used for transposon tagging and the other was that BO males were infertile and the hermaphrodites were temperature-sensitive sterile mutants (Riddle *et al*., 1996). Many earlier studies were carried out by crossing of the Bristol and Bergerac strains to create the first recombinant inbred lines (RILs) in *C. elegans*. The BO strain was however, very unstable and, unlike N2, is no longer used in research. In the past, not much attention was given to other wild isolates, this was potentially due to difficulties with finding nematodes in the wild. This is now changing and more of the research is carried out on freshly derived wild isolates (for example see Volkers *et al.,* 2013 and Stastna *et al*., 2015), because in order to understand an organism in depth one must unravel details of its natural ecology (Hodgkin and Doniach, 1997).

1.11.2 Ecology of *C elegans*

In the wild, *C. elegans* is found worldwide in predominantly humid temperate areas in microbe-rich habitats where it shares its food source with other major model organisms; *Drosophila melanogaster* and *Saccharomyces cerevisiae* (Felix and Braendle, 2010; Kiontke *et al*., 2011). Worms extracted from natural habitats used to be predominantly found in the dauer diapause

stage as the wrong substrates were sampled. However, new insights into the worm's ecology enabled the identification of growing populations and the extraction of all life stages (Dirksen *et al.,* 2016). Research into *C. elegans'* preference to different bacterial food has so far been rather modest. To date, there are limited studies have sampled their native habitats to characterize their microbiomes. The latest of such studies has discovered that the *C. elegans* natural environment is dominated by proteobacteria such as Enterobacteriaceae and members of the genera *Pseudomonas, Stenotrophomonas, Ochobactrum* and *Sphingomonas* (Dirksen *et al.,* 2016). As well as being a food source for the worm, bacteria such as *Enterococcus faecalis* and *Staphylococcus aureus* can cause lethal infections (Garsin *et al.,* 2003). In one study that looked into the behavioural aspects of food choice, *C. elegans* preferred two strains of bacteria *Bacillus mycoides* and *Bacillus soli* in comparison to the standard laboratory *E. coli* OP-50 strain (Abada *et al*., 2009). Other research has tested behavioural choices in food-seeking strategies with the results suggesting that *C. elegans* is an opportunistic feeder. This means that when given choice of various bacteria, it will always seek the highest food quality that better supports growth and reproduction (Shtonda and Avery, 2006). In another study it was discovered that N2 is longer lived when fed *Bacillus subtilis*, a common ground bacterium that *C. elegans* is likely to feed on in the wild (Garsin *et al*., 2003). In a more complex study, worm populations fed a mixture of 14 bacterial isolates exhibited enhanced population growth compared to the *E. coli* controls (Dirksen *et al*., 2016), indicating that much remains to be understood about the relationship between *C. elegans* and the bacteria it is associated with.

In general, worms are exposed to complex microbial communities in the wild and their diet is likely to include many different bacteria. This is a stark contrast to lab culturing where *C.*

elegans is kept on the *Escherichia coli* strain OP50 with any contaminating microbes removed by hypochlorite treatment (Stiernagle, 2006). *E. coli* OP50 is an uracil auxotroph, this means this strain cannot synthesize particulate organic compounds which results in limited growth on nutrient agar plates. *E. coli* OP50 has therefore been the standard lab food for *C. elegans* since being introduced by Sydney Brenner (Brenner, 1974). Although widely used, researchers have discovered that live *E. coli* OP50 can sometimes survive grinding in the pharynx and can proliferate in the intestine leading to a constipation phenotype (Garigan *et al*., 2002; Felix and Braendle, 2010). Worms freshly derived from the wild contain a variety of microorganisms in their guts (Felix and Braendle, 2010; Petersen *et al*., 2014). This varied microbiome can be an effective barrier against fungal infections (King *et al.,* 2016). These examples make a strong case that understanding the natural microbiome can unravel *C. elegans* biology in general. Innate social behaviour that can be observed in many different species of animals and in some social bacteria and amoebae can also be observed in *C. elegans* (de Bono and Bargmann, 1998). *C. elegans* is an excellent organism to study natural variation in behaviour because of its simple nervous system, good knowledge of genetics and wide availability of wild isolate strains (Hodgkin and Doniach, 1997). Investigation of genetic variability in social feeding behaviour indicates that there is variation between *C. elegans* strains: the commonly used lab strain N2 is a solitary feeder when food is abundant whilst many wild isolates display an aggregation behaviour on food when there is a plentiful supply of bacteria (de Bono and Bargmann, 1998). This behavioural difference in feeding can be assigned to alteration of a single neuropeptide receptor, *npr-1*. IMS-induced mutations in *npr-1* causes solitary N2 to feed in a social manner and converts wild types into solitary behaviour pattern (de Bono and Bargmann, 1998). These findings suggest that a single gene mutation can alter the behaviour of wild and laboratory strains. The importance of behavioural processes in nature were highlighted by response of *C.*

elegans to pathogenic bacteria as shown by Reddy *et al*., 2009. Bacteria in the wild vary greatly in the chemicals they secrete and excrete, and in their attractiveness to worms. It is therefore plausible that the behaviours observed in the laboratories is only a margin of wide behavioural choice which is displayed by the species in the wild.

Development of *C. elegans* is typically studied as a continuous process under laboratory conditions. *C. elegans* larvae hatch at the L1 stage and will arrest at this stage if there is no food, post-embryonic development only proceeding in the presence of food (Fukuyama *et al.,* 2006). When food is located, the L1 starts feeding and progresses into the L2 stage. At this stage it can either keep developing to the L3 stage or became a dauer larvae. A dauer larvae is thought to be the main dispersal morph in *C. elegans*, is an alternative developmental stage where the worm is non-feeding, long-lived and possesses increased stress resistance (Hu, 2007). Dauer larvae form under conditions that are unsuitable for growth and reproduction, with the perceived environmental stresses including high population density, low or depleted food supply and ambient temperature (Hu, wormbook, 2007). The transition to dauer arrest has been studied in order to uncover regulatory mechanisms that govern morphological change during development as well as gaining information on obligate dauer-like development which is used by many parasitic nematodes (Gottlieb and Ruvkun, 1994; Meléndez *et al*., 2003; Harvey *et al*., 2009; Green *et al*., 2013; Hu, wormbook, 2007). When conditions become favourable again, dauer larvae will develop into the L4 stage and continue development into adults. Adult lifespan varies greatly between strains, but averages 18 days under lab conditions (Stastna *et al*., 2015). However, animals in the wild often develop under variable and stressful conditions and it is likely that lifespan will be much reduced under such conditions. To date,

only one study has attempted to determine this, with culture of worms in soil with a native micro fauna showing a 10-fold reduction in lifespan (Voorhies *et al*., 2005).

1.12 Quantitative genetics

Quantitative genetics is the study of the inheritance of traits that show a continuous distribution of phenotypes in segregating populations. Traits that are controlled by many genes exhibit quantitative inheritance as each gene segregates in a Mendelian fashion. However, most traits vary quantitatively and have a complex inheritance among populations (Barton and Keightley, 2002). Mapping quantitative trait loci (QTL) can help to unveil the genetic mechanisms underlying quantitative phenotypic variation and the main approaches to identify QTLs are linkage analysis and association mapping (Mackay *et al*., 2009). QTL mapping, in its most basic form, links the two types of information – phenotypic data and genotypic data – with the key to understanding the significance of QTLs being to understand the nature of inherited variation (Barton and Keightley, 2002). The traditional approach for finding QTLs underlying natural phenotypic variation is to analyse a large number of progeny from two-parent crosses (Brem *et al.,* 2002). Although such studies have proven very useful in mapping causative loci (Brem *et al*., 2005; Smith and Krugglyak 2008), single cross experiments using common laboratory strains are limited by low genetic variation from the two parental genotypes (Cubillos *et al*., 2011). In the last 15 years the substantial increase of marker density in model organisms, driven by advances in cost-effective and rapid genotyping methods, have revolutionized genomic and transcriptomic approaches to biology (Davey *et al*., 2011). These advantages have allowed for multi-parent mapping panels to be created and analysed in many model organisms. To date, no such panel has been created or analysed in *C. elegans*.

Historically the literature pertaining to natural variation in *C. elegans* dates back to early work by T. E. Johnson (Johnson and Wood, 1982; Johnson, 1986; Johnson 1987). Early approaches to studies in natural variation were non-uniform, *i.e.* carried out at different temperatures, media and strains. Many studies were also carried out using strains that are now known to be unsuitable, such as crosses of Bristol and Bergerac (Johnson & Wood, 1982). In this study, some of the first recombinant inbred lines (RILs) were created to study variation in lifespan. The work concluded that lifespan variation in *C. elegans* has a substantial genetic component and this organism promised opportunities for selective breeding of long-lived strains to facilitate the analysis of senescence (Johnson & Wood, 1982). In later work, Johnson showed that there is no correlation between the length of the reproductive period and lifespan and concluded that the genes specific for the reproductive period are independent of these specifying the length of life (Johnson, 1987). Even some of the later work on quantitative genetics in *C. elegans,* was carried out on N2/BO crosses. One study testing life-history pleiotropy, uncovered several QTLs for fertility and body size, this suggested pleiotropic or closely linked effects, however, further analysis identified that these QTL arose due to mutations that must have occurred during the laboratory history of N2 and BO (Knight *et al*., 2001). These earlier studies did not analyse the whole genome due to the number and type of markers used. Also the N2 and BO inbred panel approach suffers from low vitality due to deleterious effect of transposon markers (i. e. transposon markers are still active thus disturbing the stability of genotypes; Hodgkin and Doniach, 1997). For this reason the Bergerac strain is no longer used in quantitative genetics, however the polymorphisms induced by the Tc1 transposon were one of the first ways that genetic differences between strains of *C. elegans* were classified (Hodgkin and Doniach, 1997). This study was therefore the first in the field that highlighted the contrast between understanding *C. elegans* as an experimental model and understanding its natural environment

and ecology (Hodgkin and Doniach, 1997). Genotyping various wild isolates by molecular markers and Tc1 revealed a wide range of differences between isolates and little polymorphism within them, a combination that allowed for the first phylogeny of isolates. It also addressed the relationship between different natural isolates and defined some distinctive differences in copulatory plug forming (Hodgkin and Doniach, 1997).

More recently, much quantitative genetics work in *C. elegans* has focussed on N2 and on the Hawaiian isolate CB4856. A good example of this approach is that from the Kammenga laboratory, where a new panel of RILs generated by crossing the canonical N2 (Bristol) strain with CB4856 has been extensively analysed over a number of years (Gutteling *et al*., 2007, Doroszuk *et al*., 2009; Rodriguez *et al.,* 2012). They found different correlations at different temperatures and located loci responsible for control of these correlations. To speed up mapping in these RILS, new N2/CB4856 introgression lines (ILs) were created with a proportion of the CB4856 segments that in most lines did not exceeded 3% (Doroszuk *et al.,* 2009). These lines identified novel loci underlying natural variation for two traits and resulted in in QTLs with a lifespan shortening effect on the CB4856 allele (Doroszuk *et al*., 2009). An alternate set of lines also derived from N2 and CB4856 has been created by the Kruglyak lab with an advanced intercross design and a large number of RILs used to maximise mapping power (Li *et al*., 2006; Rockman and Kruglyak, 2008; Andersen *et al.,* 2015).

Lines such as these derived from N2 and CB4856 have been very successful for QTL and gene mapping, but are still constrained by low variation as these two common laboratory strains only capture a small fraction of the genetic and phenotypic variation that is present in natural populations. As natural wild isolates that are adapted to different niches, they may also represent a better resource for natural variation studies because their alleles were selected in

specific environments (Carreto *et al*., 2008). Also studying natural genetic variation is crucial for understanding gene functions as allelic interactions in divergent genetic backgrounds does play an important role in determining the genetic basis of complex traits (Kammenga *et al*., 2008). These arguments create a need for mapping in multi-parent lines derived from wild isolates currently absent in *C. elegans* research.

1.13 Ageing pathways

The lifespan of an animal is regulated by diverse genetic and environmental factors (Kenyon, 2005) with many of the crucial mechanisms evolutionary conserved across species (Curran and Ruvkun, 2007). Mutations that postponed senescence were first discovered in worms and led to the discovery of the evolutionary conserved Insulin/Insulin-like (ILS) growth factor signalling pathway (Figure 1.1; Panowski and Dillin, 2009). In *C. elegans* this pathway is central to both growth and metabolism (Tullet *et al.,* 2008). It has been observed that this pathway has a conserved role in ageing in *C. elegans,* is important in the characterization of downstream elements of metabolic signalling (Gems and Partridge, 2013) and that mutations in the insulin/ILS pathway can alter the process of ageing (Kenyon, 2010b). Research into regulatory mechanisms can reveal the exact pathways necessary to postpone senescence and minimize age-related diseases (Kenyon, 2010a). In *C. elegans*, lifespan extension seems to be partly the consequence of a reduction in the activity of the ILS pathway and requires the forkhead transcription factor *daf-16* (FOXO-family transcription factor; Kenyon *et al*., 1993). This pathway is evolutionary conserved and functions to modulate wild-type longevity, metabolism and stress resistance (Gami and Wolkov, 2006). Insulin/IGF-1-like signalling pathway exerts its effect on the animal by changing downstream gene expression (Figure 1.1; Murphy *et al*.,

2003). DAF-2/Insulin-like receptor recruits AGE-1 (phosphoinositide 3-kinase/PI3K, p110 catalytic subunit) to the cell membrane. AGE-1 generates phospholipid signals, PIPs (minor phospholipid components of cell membrane), which in turn activate the AKT-1 -2(serine/threonine kinase AKT/PKB), PDK-1 (3-phosphoinositide-dependent kinase) and SGK-1 (serine/threonine protein kinase). These phosphorylate DAF-16/FOXO (transcription factor of the HNF-3/forkhead family, homologous to human Forkhead Box O) preventing nuclear translocation, thereby blocking the transcription of its target genes (Wolff and Dillin, 2006).

Reduced insulin-signalling results in the activation of DAF-16/FOXO, which in turn enters the nucleus where it induces gene expression that leads to increased lifespan and stress resistance (Murphy, 2006). DAF-18/PTEN (lipid phosphate, homologous to human PTEN tumour suppressor) is the negatively regulated target of the DAF-2/AGE-1/AKT-1/2/PDK-1 signalling cascade. DAF-18/PTEN down-regulates AGE-1 signalling by dephosphorylating PIPs (Gami and Wolkov, 2006) and therefore regulates metabolism dauer arrest and longevity (Ogg and Ruvkun, 1998). In addition to regulating lifespan, the insulin/IGF-1 pathway also regulates the entry into the dispersal morph like state dauer (Guarente and Kenyon, 2000). Given the plethora of research in which either N2 or its derived mutants were used for the identification of functional components of the ILS pathway, the question is, would the use of new RILs panel constructed from the wild isolates identify known genes of the ILS pathway or would it uncover previously undetected genes that potentially play a role in the wild.

The other major signalling pathway that determines longevity in C. elegans is the mTOR pathway. mTOR is a nutrient sensing pathway and a major mediator of growth and reproduction in response to the availability of growth factors and amino acids (Honjoh *et al*., 2009). Inhibition of the mTOR signalling pathway at several points promotes life extension in

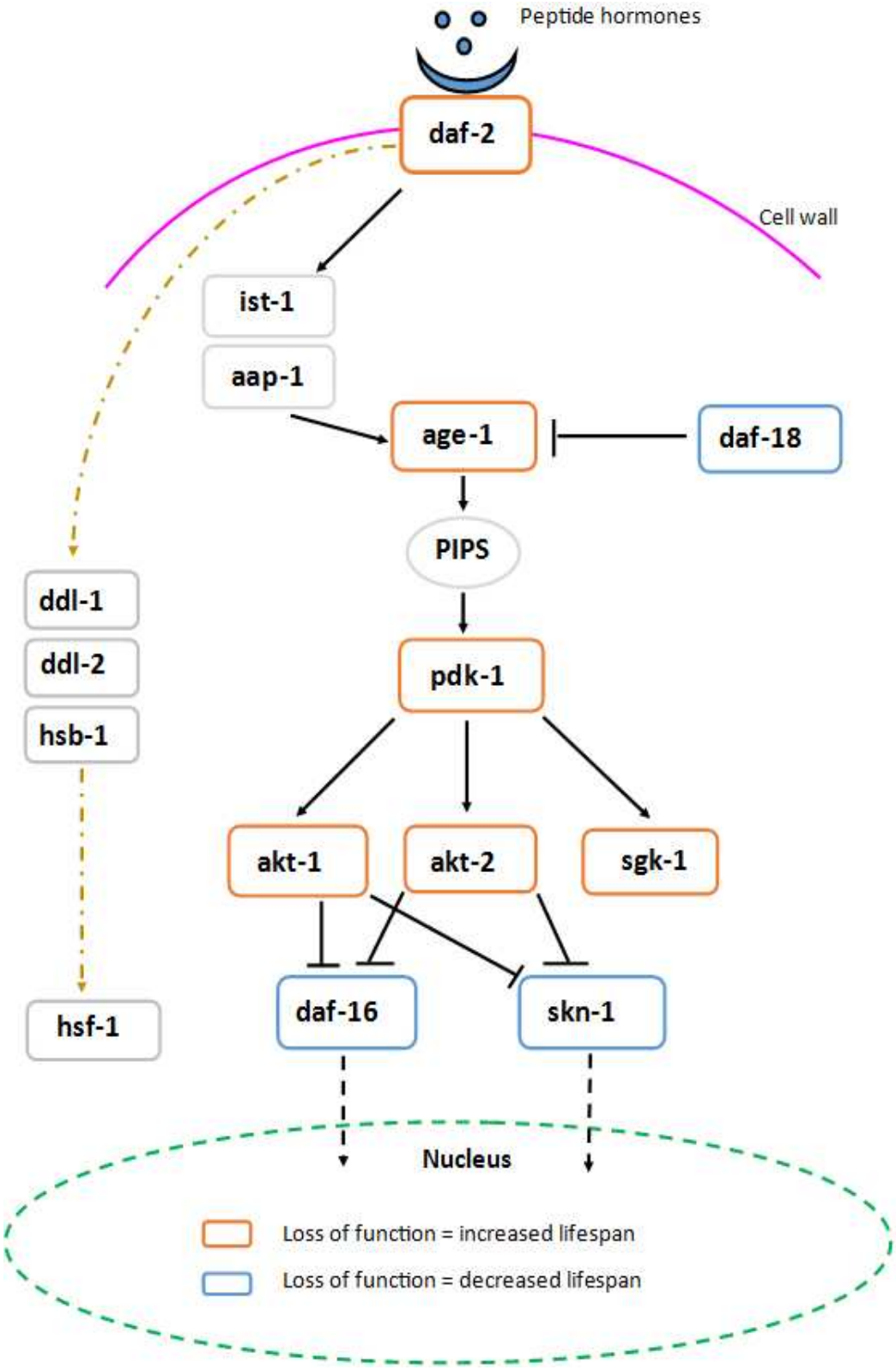

Figure 1.1 Schematics of Insulin/insulin-like growth factor signalling (ILS) pathway in *C. elegans*. Activated ILS pathway exert its biological effect by downstream signalling preventing or allowing of daf-16 to translocation to the nucleus.

worms (Hansen, 2008) and also extends longevity in a variety of other species such as S. cerevisiae, C. elegans, D. melanogaster and M. musculus (as reviewed in Kapahi *et al*., 2010).

The mTOR signalling network exists in two complexes, mTORC1 and mTORC2. These complexes react with different coactivators. mTORC1 is thought to interact with DAF-15/Raptor (mTORC binding partner Raptor), RAGA-1, RAGC-1 and RHEB-1/Rheb to impair activity of DAF-16 (reviewed in Zoncu *et a*l., 2011). mTORC1 and mTORC2 inhibit expression of SKN-1/Nrf (transctibtion factor, accumulates in the nuclei in response to oxidative stress). DAF-16 and SKN-1/Nrf are required for lifespan extension. It is not yet clear which of the mTOR complexes regulate PHA-4/FOXA (transcription factor FOXA) which is also required in extension of lifespan (Sheaffer *et al*., 2008).

1.14. Aims of the Thesis

The aims of this thesis are to explore the impact of natural variation on lifespan and stress responses in *C. elegans*. This work differs from the standard quantitative mapping in *C. elegans* by using a new panel of 200 sequenced genotyped recombinant inbred lines (RILs) derived from four freshly collected wild isolates: JU1511, JU1926, JU1931 and JU1941. These strains are representative of genotypically distinct groups of *C. elegans* isolates from two different sites in France and are distinct from both N2 and CB4856. The new panel should therefore be useful in determining the genotype-phenotype relationship in the absence of laboratory derived alleles and be more indicative of subtle allelic effects in wild populations.

Aims of the Thesis:

1. To investigate previously isolated lifespan QTLs detected in N2/CB4856 introgression lines (Doroszuk *et al*., 2009) in order to determine if they can be reproduced, how they respond to dietary restriction and if they can be explained as a consequence of maternal hatching (this work will be an expansion of my undergraduate study). To compare these results, similar assays will be undertaken in N2/CB4856 RILs, in selected wild isolates and in a panel of complimentary CB4856/N2 introgression lines (Chapter 2).
2. To review the effects of various DR treatments in a wide range of model and non-model organisms to investigate the extent to which the effects of DR in different genetic backgrounds support the contention that DR represents a universal means of extending lifespan (Chapter 3).
3. To map lifespan under normal and mild dietary condition, as well as survival under oxidative stress, cold shock and heat shock in a new panel of 4-parent RILs in order to determine the extent to which results are comparable to those from studies on N2 and CB4856 (Chapters 4 and 5) and to explore specific QTLs (Chapter 6).
4. To review various dietary regiments in *C. elegans* such as total starvation, bacterial dilution and peptone withdrawal and to compare the effects of different methods of DR in various genetic backgrounds (Chapter 7).

CHAPTER TWO: Natural variation in the effects of dietary restriction on lifespan in *Caenorhabditis elegans*

Work reported in this chapter has been published as:

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Abstract

Dietary restriction (DR) appears to act as a general non-genetic mechanism that can robustly prolong lifespan. There have however been reports in many systems, of cases where DR either shortens, or does not affect, lifespan. Here we analyze lifespan and the effect of DR on lifespan in wild isolates and introgression lines (ILs) of the nematode *Caenorhabditis elegans.* These analyses identify genetic variation in lifespan, in the effect of DR on lifespan and also in the likelihood of maternal, matricidal, hatching. Importantly, in the wild isolates and the ILs, we identify genotypes in which DR reduces lifespan. We also identify, in recombinant inbred lines, a locus that affects maternal hatching, a phenotype closely linked to DR in *C. elegans*. These results indicate that, as has been seen in other systems, DR has genotype-dependent negative effects in *C. elegans*, reducing lifespan in some genotypes.

2.1 Introduction

Ageing is a universal phenomenon during which progressive changes ultimately lead to death. Lifespan can be affected by many factors, one being DR. DR, or caloric restriction, is a reduction in caloric intake without malnutrition. This non-genetic intervention can robustly extend lifespan in variety of species from yeast to mice for example (McCay *et al*., 1935; Mair *et al*., 2005; Lee, 2006; Mair and Dillin, 2008, for review see Fontana, 2010). In a number of model species, DR has also been shown to delay the onset or severity of age-related traits that are associated with diseases such as diabetes, dementia and cardiovascular diseases (Mair and Dillin, 2008; Fontana, 2010; Herndon *et al.*, 2002 and Kaufman *et al*., 2010). Studies in primates also suggest that DR can improve health in rhesus monkeys (Mattison *et al*., 2012 and Colman *et al.,* 2009), although the effect on lifespan is less clear, with one study reporting a reduction in mortality (Colman *et al.,* 2009), while a second observed an improved health span and no effect on lifespan (Mattison *et al.,* 2012). Whilst the effects of DR on human lifespan are still unknown, the accumulating data indicates that DR without malnutrition improves markers of aging (for review see Cava and Fontana, 2013). More studies are however needed to allow for a better understanding of the molecular processes that play a part in individual genotype-dependent responses to DR in humans.

Despite the general case for DR prolonging lifespan, examples exist in yeast (Schleit *et al.,* 2013), worms (Sutphin and Kaeberlein, 2008), fruit flies (Carey *et al.,* 2002), house flies (Cooper *et al.,* 2004), rats (Barrows and Roeder, 1965) and mice (Silberberg and Silberberg, 1954; Harper *et al.,* 2006 and Liao *et al*., 2010) where DR either has no effect on, or even reduces, lifespan. Some of this variation is a consequence of different methodologies and of the severity of the DR. For example, Mediterranean fruit flies showed no increase in longevity with moderate DR

and dramatically increased mortality once the level of DR reached 50% (Carey *et al.,* 2002). Genetic background is also an important factor. For example, no effect of DR was detected in freshly caught wild mice maintained on a regime that prolonged the lifespan of lab adapted animals (Harper *et al*., 2006). Similarly, changes in lifespan in response to DR were variable between recombinant inbred mouse strains (Liao *et al.,* 2010), with DR shortening lifespan in more of the strains than which it increased lifespan. Variable responses to DR have also been observed in a genetically heterogeneous population of budding yeast (*Saccharomyces cerevisiae*), with the effect of DR varying from a 103% increase to a 79% decrease among strains yeast (Schleit *et al*., 2013).

Invertebrate species provide an excellent model for ageing research due to their relatively short lifespan and experimental tractability. One such model system is the nematode *Caenorhabditis elegans*. The effects of DR on lifespan in *C. elegans* have been investigated via several methods including the use of mutants (e.g. *eat-2*) deficient in pharyngeal pumping (Guarente and Kenyon, 2000), axenic culture (Houthoofd *et al*., 2002), bacterial dilution in liquid cultures (Bishop and Guarente, 2007), dilution or total withdrawal of peptone from the agarose plates (Hosono *et al.,* 1989), the use of UV-killed bacteria (Greer *et al.,* 2007) and total starvation (Lee *et al.,* 2006; Kaeberlein *et al*., 2006). All of these experimental manipulations have been successful in prolonging lifespan (Greer and Brunet, 2009). In *C. elegans*, the longevity response to DR is actively regulated through independent and overlapping pathways. These pathways are evolutionary conserved and include; mechanistic target of Rapamycin (mTOR) for chronic food limitation (strong-effect *eat-2* mutants and starvation treatments, Hansen *et al*., 2007), AMP-activated protein kinase (AMPK) (bacterial dilution on plates starting in middle age and peptone dilution, Greer *et al*., 2007), sirtuins (mild DR or weak-effect *eat-2* mutants, Rogina

and Helfand, 2004) and insulin/insulin-like growth factor (IGF-1) signaling (intermittent feeding, Honjoh *et al*., 2009).

In addition to the effects on lifespan, *C. elegans* responds to DR by increasing the rate of maternal hatching, a situation where fertilized eggs hatch inside the reproductive tract and the resulting progeny consume the mother. Maternal hatching in *C. elegans* is also associated with ageing, with the rate increasing with age over the reproductive period. Increased maternal hatching in response to DR may therefore represent the effects of stress or damage, or may be an adaptive parental response to starvation (Chen and Caswell-Chen, 2004; Chen and Caswell-Chen, 2003). Many ageing and most DR studies in *C. elegans* have avoided maternal hatching by assaying worms in the presence of 5-fluorodeoxyuridine (FUdR), a drug that inhibits cell division and prevents eggs from hatching. This is however potentially problematic when considering variation in the response to DR, as FUDR has been shown to affect lifespan in a genotype-dependent manner (Van Raamsdonk and Hekimi, 2011; Davies *et al.,* 2012) and to interact with the stress response (Feldman *et al.,* 2014). The genetics of variation in maternal hatching is also poorly understood and, although a small number of quantitative trait loci (QTL) affecting maternal hatching have been identified (Snoek *et al.,* 2014), it is not known if these are related to variation in lifespan.

There is extensive genetic variation between wild isolates of *C. elegans* (Andersen *et al*., 2012; Volkers *et al.,* 2013 and Thompson *et al*., 2013), but these have only sparsely been studied and nearly all *C. elegans* research is carried out on the N2 (Bristol) strain and its derived mutants. Natural variation in *C. elegans* lifespan has however been analyzed, principally using RILs and subsequent QTL mapping (for review see Shmookler Reis *et al.,* 2006). More recent work using ILs derived from wild-types N2 and CB4856 has identified novel loci underlying natural variation

in two age-related traits (pharyngeal pumping and lifespan; Doroszuk *et al*., 2009) and age related expression QTLs (Viñuela *et al*., 2010). It is however unknown if these QTLs also affect the response to DR. These ILs lines were constructed with N2 background and small introgressions of CB4856. The majority of analyses of DR in *C. elegans* have been undertaken in the N2 genetic background, with one study analysing five wild isolates of *C. elegans* and two of *C. remanei* (Suptin and Kaeberlein, 2008) and identifying variation in response to DR by bacterial dilution, a method that results in maximal lifespan extension from DR in *C. elegans* (Lee, 2006 and Kaeberlein *et al.*, 2006). In this study, DR treatment extended lifespan for all *C. elegans* isolates and the mean lifespan was extended for one isolate of *C. remanei* (Suptin and Kaeberlein, 2008). To address these issues, here I have a) analyzed the effect of mild DR on lifespan and maternal hatching in five N2/CB4856 ILs; b) mapped variation in maternal hatching in N2/CB4856 RILs; c) measured lifespan in complimentary panel of brand new CB4856/N2 RILs and d) compared these data to the effects of DR on wild isolates. We show that maternal hatching is a genetically variable phenotype and linked loci can be found by QTL analysis. We also conclude that the effect of DR on lifespan is polygenic, does not exclusively prolong lifespan and depends on the genetic background.

2.2 Methods

2.2.1 Worms

Worms used were N2 (Bristol), the N2/CB4856 ILs ewIR1, ewIR18, ewIR21, ewIR40 and ewIR51 (Doroszuk *et al.,* 2009), N2/CB4856 RILs (Viñuela *et al*., 2010; Li *et al*., 2006; Viñuela *et al*., 2012; Elvin *et al*., 2011; Rodriguez *et al.,* 2012 and Li *et al*., 2010), CB4856/N2 RILs (unpublished data Sterken) and wild isolates (Volkers *et al*., 2013; donated by M.A. Felix to JK). The selected ILs

contain five of six previously identified QTLs with potential life-shortening effects of the CB4856 allele lifespan (Doroszuk *et al*., 2009). An additional lifespan QTL identified in these lines on chromosome X was not retested here as the QTL region contains N2 version of *npr-1* (The aallelic variation in *npr-1* in N2 has been described that influences several behaviors). As variation between N2 and CB4856 at *npr-1* affects many behavioral traits in *C. elegans*, which in turn effect food availability (Green *et al*., 2013 and Andersen *et al.,* 2014), this QTL was not analyzed in this study. All lines were maintained at 20°C on standard nematode growth medium (NGM) with *Escherichia coli* OP50 as a food source (Stiernagle, 2006).

2.2.2 Lifespan assays CB4856/N2 RILs, N2/CB4856 ILs and wild isolates.

Worms were grown *en masse* to adulthood and eggs were collected from sodium hypochlorite treated gravid adults (Stiernagle, 2006). These eggs were then maintained for 24 hours without food at 20°C to synchronize the population, synchronized larvae stage 1 worms (L1s) then transferred to fresh 35mm plates (n = 40 per treatment per genotype, with 1 worm per plate for the ILs, 10 worms per plate for the RILs and 5 worms per plate for the wild isolates.) The Different number of worms on plates were due to trialing out the peptone withdrawal technique. I used 1 worm per plate for the ILs to avoid starvation effect. However, the peptone withdrawal is a mild DR method and I could therefore place 10 worms per plate for RILs worms. Culturing of wild isolates is complicated, as most lines do not like to be on crowded plates therefore only 5 worms were used for wild isolates. Control, *ad libitum* food, worms were maintained on standard NGM plates and DR was performed by total peptone withdrawal from standard NGM plates (Hosono *et al.*, 1989 and Harvey *et al.*, 2008), a condition that stops bacterial growth on the plates. Worms were observed daily, with nematodes transferred to new plates every day until reproduction had ceased. Worms were considered to have died if they

were not moving and failed to respond to touch with a worm-pick. Any worms that died due to maternal hatching (bagging) were noted, and these data were used to investigate variation in maternal hatching in the ILs and wild isolates. As is routine for the analysis of lifespan in *C. elegans* deaths by maternal hatching were censored out of the analysis of lifespan.

2.2.3 Maternal hatching in the RILs

To assay maternal hatching in the RILs, 40 synchronized worms per RIL were followed for 1 week after the L4 stage and the number of maternal hatchings recorded daily.

2.2.4 Data analysis.

Lifespan in the ILs was analyzed using Kaplan-Meier Survival curves with significance determined by log-rank analysis as is standard way for lifespan assays (in Minitab® Statistical Software; Mintab Ltd., Coventry). QTL mapping of the percentage maternal hatching per RIL was done using a single marker model (Snoek et al., 2014). Data is archived in WormQTL (http://www.wormqtl.org) (Snoek *et al.,* 2013; Van Der Velde *et al*., 2014 and Snoek *et al*., 2014).

2.3 Results

To test if the genetic background affects the lifespan effects of DR we tested five ILs, each containing no more than 3% of an introgressed portion of the CB4856 genome in an N2 genetic background that had previously been shown to affect lifespan (Doroszuk *et al.,* 2009). Overall, DR increased mean lifespan ($p = 0.005$) in the ILs tested (Table 2.1).

	Lifespan per strain (days)				Maternal hatching per strain			
Line	NGM	DR	effect	p-val (NGM vs DR)	NGM	DR	effect	p-val (NGM vs DR)
N2	17.7	22.2	23.50%	<0.0001	5.10%	0	-5.10%	NS
ewIR01	15.6	22.5	41.10%	<0.0001	2.60%	0	-2.60%	NS
ewIR18	17.7	20.8	17.10%	<0.01	5.10%	5.40%	0.30%	NS
ewIR21	14.2	13.2	-2.50%	NS	18.90%	33.30%	14.40%	<0.01
ewIR40	17.1	14.2	-14.90%	<0.02	18.40%	27.00%	8.60%	<0.01
ewIR51	15.5	13.1	-6.40%	NS	10.00%	41.70%	31.70%	<0.05

Table 2.1 Lifespan and the effect of DR in ILs and N2. Shown are the mean lifespan and the percentage of worms that died due to maternal hatching under *ad libitum* (nematode growth medium plate NGM) and DR conditions, the percentage effect of DR on the trait and the significance. Introgression shows the chromosome and maximum limits (i.e. the position of the flanking N2 markers) of the portion of the CB4856 introgressed into this IL.

However, large differences between the genotypes were observed (p = 0.007). In N2 and two ILs (ewIR01 and ewIR18) DR prolonged mean lifespan. However, DR had no effect on lifespan in two ILs (ewIR21 and ewIR51) and reduced lifespan in another (ewIR40). This indicates that, in the ILs tested, the effects of DR are dependent on genotype or prevalence of maternal hatching.

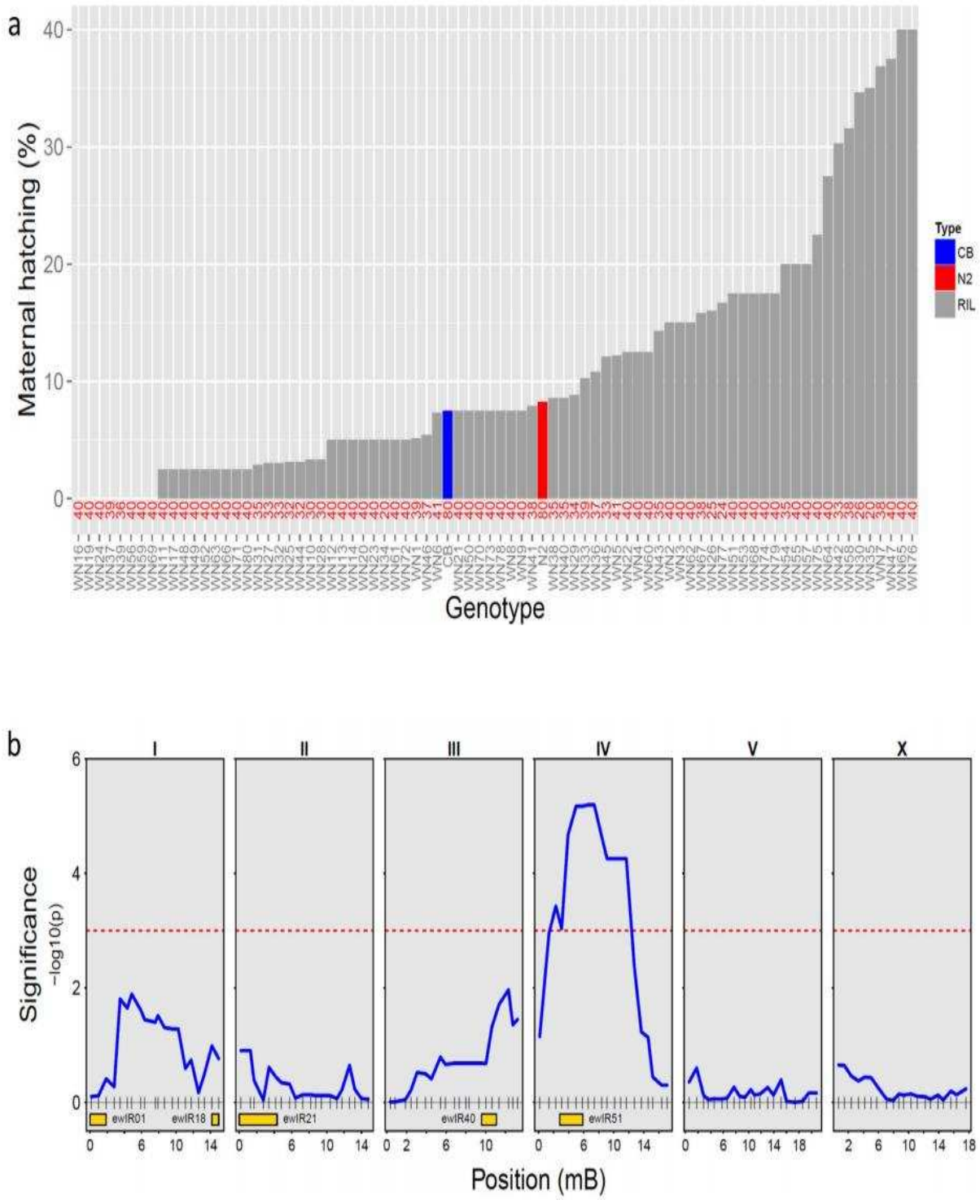


Figure 2.1 Maternal hatching in N2/CB4856 RILs. a) Percentage of maternal hatching in N2/CB4856 RILs, CB4856 (blue) and N2 (red), this plot identifies transgressive segregation, i. e. the formation of extreme phenotypes compared to phenotypes observed in parental lines. The

red numbers on the x-axis indicate total number of animals per line. b) QTL profile for the percentage of worms that died due to maternal hatching. Significance (-log10(p), blue lines) plotted against the marker position in megabase pairs (grey vertical lines on the 0 line). Chromosome names are indicated above the panels. Threshold (0.05; 1000 permutations) is shown by the dotted red line.

These data also support three previously identified lifespan QTLs from N2/CB4856 ILs (Doroszuk *et al*., 2009) in lines ewIR01, ewIR21 and ewIR51 (comparisons of ILs against N2, $p < 0.05$; Table 2.1). Maternal hatching also differed among these ILs, with the genotypes for which DR prolonged lifespan (N2, ewIR01 and ewIR18) showing limited maternal hatching (Table 2.1). Other genotypes (ewIR21, ewIR40 and ewIR51) showed increased maternal hatching under both DR and control (NGM) conditions. As individuals that died via maternal hatching were not included in the analysis of lifespan data, the lack of lifespan extension in response to DR in these ILs is not a direct result of increased maternal hatching.

QTLs affecting maternal hatching have previously been identified in ewIR21 and ewIR51 (Snoek *et al.,* 2014), this analysis therefore identifies an additional QTL at the introgression of ewIR40. Moreover, the analysis shows that these QTLs also cause increased maternal hatching in response to DR. As we detected multiple shared loci involved in lifespan, the effect of DR on lifespan and maternal hatching, these results suggest a link between the lifespan prolonging effect of DR and maternal hatching.

To test if variation in maternal hatching can be linked to more loci we used RILs produced from crosses between N2 and CB4856 (Li *et al.,* 2006). We found extensive variation between the RILs and transgression in maternal hatching (Figure 2.1a). One major QTL for maternal hatching rate could be found roughly at the position of the CB4856 introgression in IL ewIR51 (Figure

2.1b; left arm CHR. IV), with this QTL explaining 22.8% of the trait variation. Taking marker 66 (at chromosome IV ~6M) as the peak of this maternal hatching QTL, we found an effect of 5.2%, such that the maternal hatching rate was 10.4% higher for the CB4856 allele than the N2 allele (Figure 2.2). This is confirmed by the 10% maternal hatching of ewIR51. The two other loci found in ewIR21 and ewIR40 (Table 2.1) were not detected in the RILs (Figure 2.1b) even though they had a larger effect and increased maternal hatching to almost double of that of ewIR51. This suggests an interaction of these two loci with the genetic background. Maternal hatching is therefore likely to be a complex polygenic trait and genetically balanced in the parental lines.

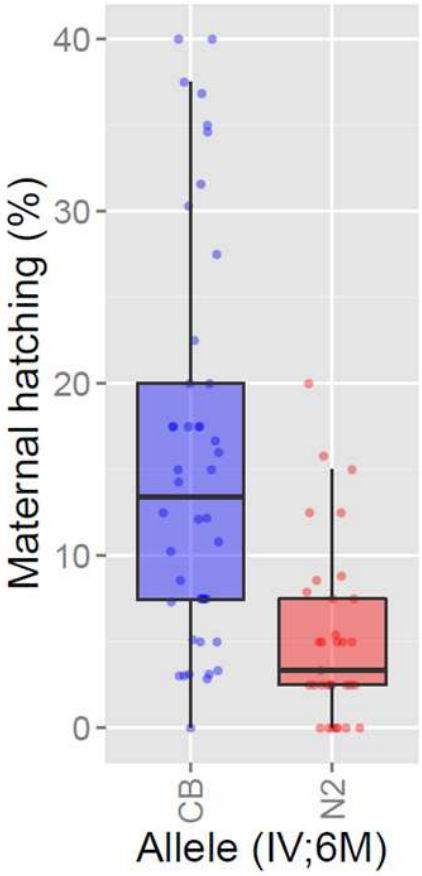


Figure 2.2 Allelic effect on maternal hatching. The maternal hatching rates of the RILs grouped by their allele on chromosome IV at ~6M basepairs, CB4856 (blue) and N2 (red).

To investigate if lifespan, maternal hatching and the effect of DR could also be under balancing selection in nature, we studied these traits in 23 wild isolates and N2 (Figures 2.3 – 2.5). Remarkably, DR reduced lifespan in about a third of the wild isolates in comparison to N2, with the effect ranging from an increase in mean lifespan in JU1937 of 4.5 days to a decrease of 2.8 days in WN2003 (Figure 2.3). From these results we can conclude that there is genetic variation for DR within C. elegans and that DR does not extend lifespan in all genotypes. We also found large variation in maternal hatching between the wild isolates, however, unlike in the ILs, this does not appear to be associated with lifespan or the effect of DR on lifespan. These results are therefore likely to reflect genotype-dependent responses to the environment.

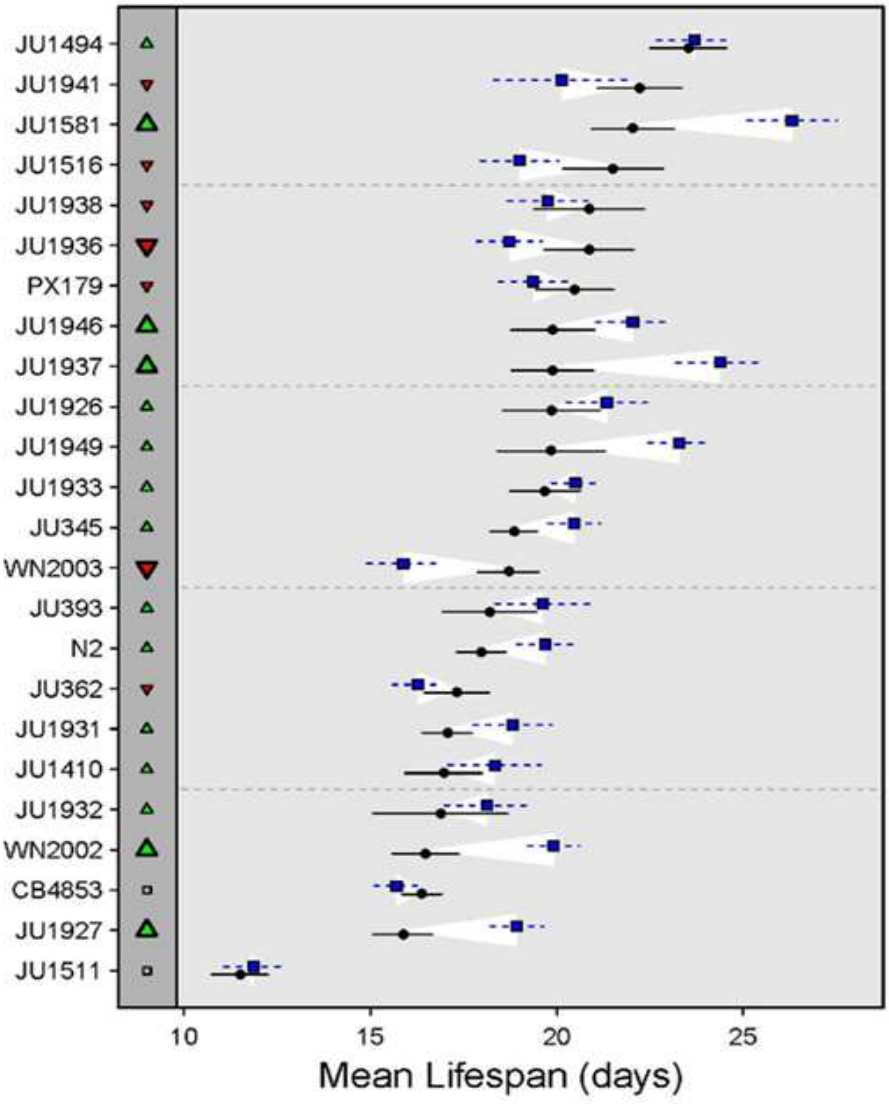


Figure 2.3 Lifespan and the effect of dietary restriction in wild isolates. Mean lifespan, ±SE, under (dots) normal (NGM) and (squares) DR conditions. The white marked regions denote the effect of DR on mean lifespan. The effect of DR on lifespan is also indicted by the triangles on the left, green triangle pointing upwards indicates a positive effect of DR and red triangle pointing downwards a negative effect of DR. Large triangles indicate a significant difference between NGM and DR.

Comparison of these results to lifespan in a new panel of complimentary ILs of CB4856/N2 indicates that average lifespan of the new panel (15.99) is very similar to that of N2/CB4856 (16.09) whilst the wild isolates are on average longer lived (18.75; Table 2.2). Heritability calculation for the wild isolates is also higher (22%) in comparison to N2/CB4856 (12.4% and 11.8%) respectively. To observe the spread of the data, histograms were plotted for N2/CB Ils and wild isolates for normal lifespan and lifespan under DR treatment (Figure 2.4). DR treatment seems to result in split of the lifespan in two observable cohorts especially for the wild isolates that can be observed as a dip at the day 20 for both DR treated cohorts.

	NGM					DR				
Genotype	Mean LS	St Dev	p-value	n	Heritability	Mean LS	St Dev	p-value	n	Heritability
N2/CB ILs *	17.58	4.305	<0.005	202	12.42%	19.85	5.106	<0.005	177	29.90%
Wild isolates	18.75	5.478	<0.005	543	22%	19.55	5.471	<0.005	599	26.60%
CB/N2 ILs	15.99	5.535	<0.005	3424	11.80%					
N2/CB ILs	16.09	2.177	0.161	88	N/A					

Table 2.2 Comparison of lifespans in ILs. N2/CB4856 ILs* represents mean lifespan for N2/CB4856 ILs (ewIR1, ewIR18, ewIR21, ewIR40 and ewIR50). P-values were calculated from one-way anova.

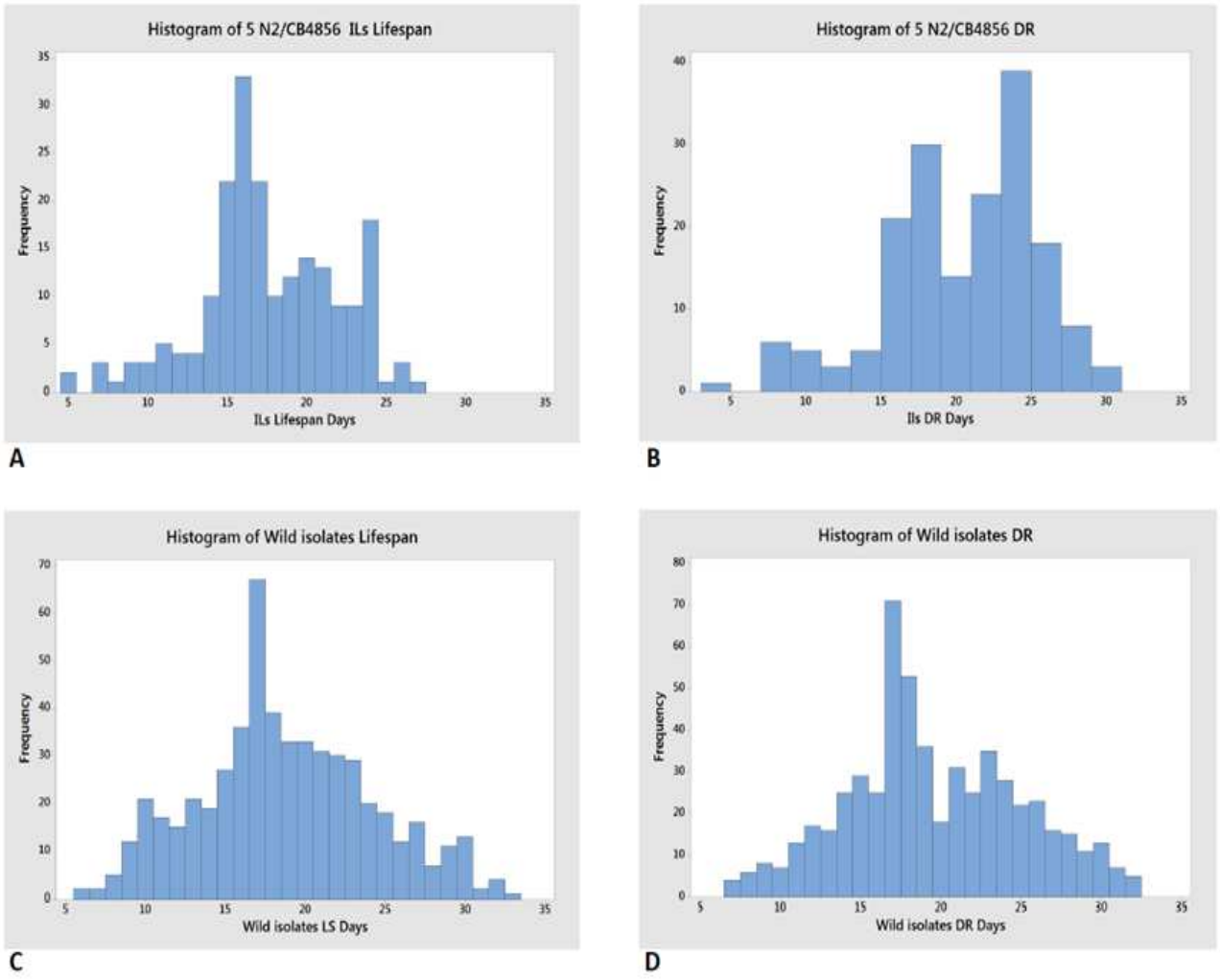


Figure 2.4 Histograms of N2/CB ILs and Wild isolates for normal lifespan and lifespan under DR treatment. Histograms were plotted from all data for the ILs and wild isolates.

Percentage of maternal hatching does not correlate between the DR and control treatment except for the lines with 40 and above percent of maternal bagging in wild isolates JU1949, JU1516, WN2003 and Ju1932 (Figure 2.5).

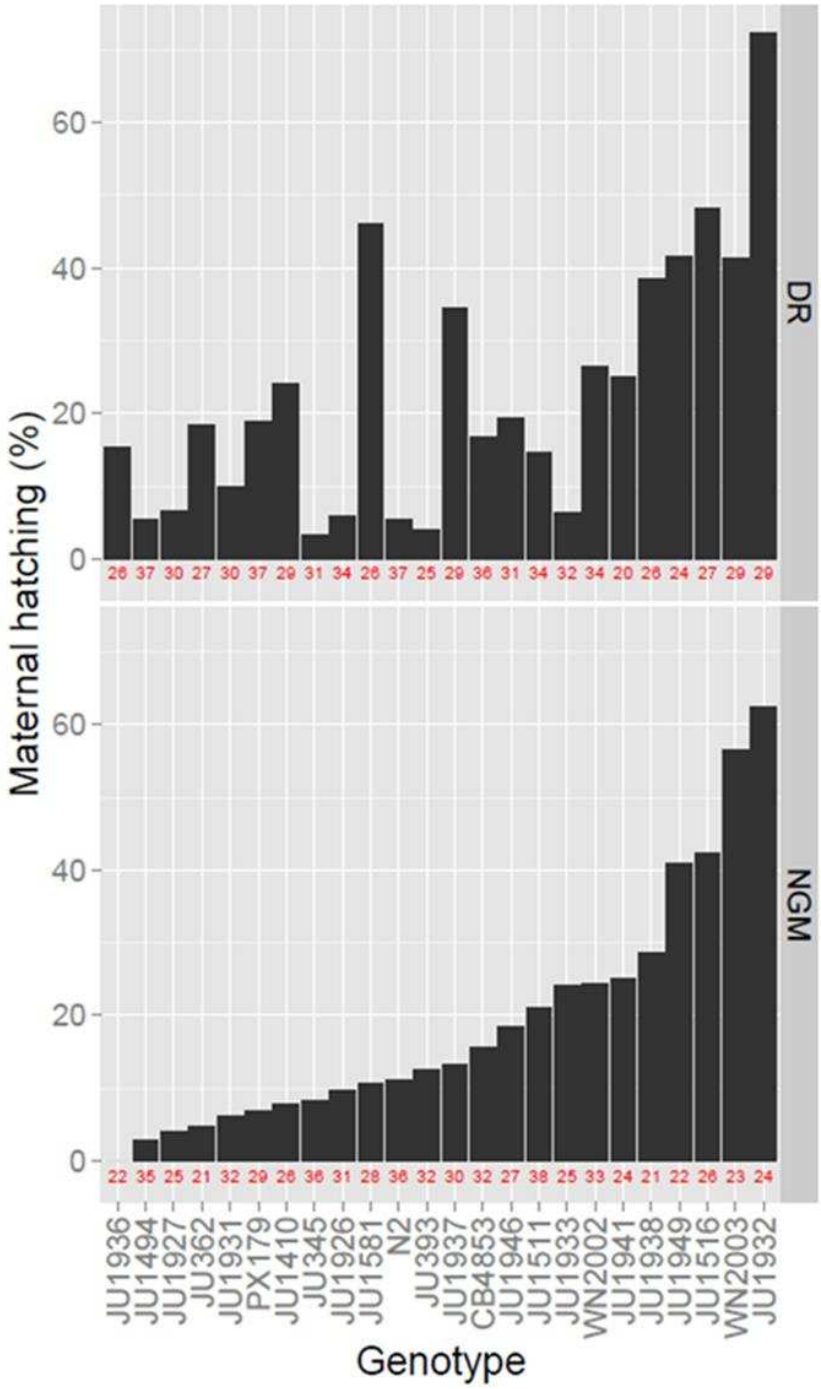


Figure 2.5 Maternal hatching rate of the wild-isolates on DR (top panel) and NGM (lower panel).

Wild isolates were ordered by their maternal hatching rate on NGM. Total number of animals tested for each wild isolate and condition are shown by the red numbers on the x-axis.

2.4 Discussion

In this study, we have focused on natural variation in lifespan, the response to dietary restriction and on maternal hatching in *C. elegans.* In ILs we identified three QTLs affecting lifespan, three affecting the response to DR and three affecting maternal hatching. This analysis confirms three (ewIR01, ewIR21, ewIR51) of five previously identified lifespan QTLs in this panel of ILs lifespan (Doroszuk *et al.,* 2009). Differences between the studies are likely to be due to either differences between the laboratories or the small population sizes used in previous analyses of lifespan (Doroszuk *et al*., 2009). Assembly of the CB4856 genome indicates that there are a number of regions of exceptionally high divergence between N2 and CB4856 (Thompson *et al.,* 2015). Comparison of the location of these regions to the limits of the introgressions in the ILs analyzed here indicates that three of the ILs (ewIR01, ewIR21, ewIR51) contain such highly diverged regions, but that ewlR18 and ewlR40 do not. Given this, the effects of these diverged regions on lifespan may therefore warrant further analysis.

We also find the ILs respond differently to DR, with DR extending the lifespan of two ILs, having no effect on two other ILs and reducing the lifespan of a fifth IL (Table 2.1). Critically, we find that DR affects lifespan in a genotype specific manner, making it likely that even mild DR will affect the detection of some lifespan QTLs. A DR regimen of total peptone withdrawal from NGM plates was chosen for its mild DR effect (Hosono *et al.,* 1989). Under this condition, the *Escherichia coli* OP50 does not grow on the plates but is not killed. Whilst the molecular mechanisms of dietary interventions are still poorly understood, it is known that various DR methods operate via independent and overlapping pathways (Greer and Brunet, 2009). For example, the low-energy-sensing AMPK is required for lifespan extension in bacterial and peptone dilution on NGM plates (Greer and Brunet, 2009).

The three ILs in which DR failed to prolong lifespan showed increased maternal hatching in both feeding regimes, with a significant increase observed in response to DR. Although the increased maternal hatching does not directly explain the lack of a response to DR, the overlap between the QTLs suggests that there is a relationship between the response to DR and the increased maternal hatching. It is likely that this is a consequence of an underlying factor affecting both traits. One possibility is that increased maternal hatching is caused by a maternal response to the DR environment which in turn leads to sacrificing themselves to allow progeny development as proposed by (Chen and Caswell-Chen, 2003). This scenario seems unlikely in this case given the variation between lines and the mild form of DR (~20%) used. An alternative possibility is that the differences are due to genetic incompatibilities between CB4856 alleles and the N2 background (Snoek *et al.*, 2014), with these incompatibilities affecting both maternal hatching and the response to DR.

Comparison of maternal hatching in the ILs and the RILs (Table 2.1 vs Figure 2.1b) indicates that the major QTL found in the RILs co-locates with one of the QTLs found in the ILs (ewIR51). The two other QTLs in the ILs were not found in the RILs. This might be due to differences in experimental settings (lab-to lab) or the more complex background of the RILs compared to the ILs. It could also be due to alleles being more balanced in the RILs preventing the maternal hatching phenotype in many combinations. This observation mirrors the observed pattern of trait variation identified in a previous comparison of ILs and RILs in C. elegans (Snoek *et al.,* 2014), with analysis of ILs revealing more loci affecting the trait than are seen in RILs. This may imply that the effects observed, particularly the observed reduction in lifespan in response to DR observed in ewIR40, are a consequence of incompatibilities between CB4856 alleles and the N2 genetic background.

Arguing against this explanation is the observed variation in lifespan, DR and maternal hatching in the wild isolates from various locations in France and The Netherlands. The genetic and gene expression differences between these wild isolates are characterized by gene-environment signatures (Volkers *et al.,* 2013), and it is therefore reasonable to expect that different isolates will display a differential response to DR treatment. Maternal hatching in wild isolates is also highly variable (Figure 2.5), DR in some cases increasing and in some decreasing the rates of maternal hatching. In most cases, DR had a life prolonging effect on most wild isolates.

Thus, the overall effect of DR is an extended lifespan. However, seven wild isolates were shorter lived under DR compared to control conditions and an additional three isolates showed negligible effect of DR on mean lifespan. Together with the results of the IL analysis, this shows that DR is not a universal method of lifespan extension in genotypes obtained from specific crosses as well as genotypes freshly taken from their natural habitat. This mirrors findings in mice and rats, where extensive variation between genotypes has been observed. In these rodent systems, a possible explanation for this variation relates to lab adaptation and the effects of inbreeding (Harper *et al.,* 2006; Liao *et al.,* 2010, also see Swindell, 2012, for discussion of this variation). Given that both wild isolates and lab isolates of *C. elegans* are highly inbred, our results are not likely to represent a lab adaptation, but could be explained as genetic plasticity in response to diminished resources rather than that of true caloric restriction. As previously stated the peptone dilution in NGM plates is relatively mild form of DR in *C. elegans* (Hosono *et al*., 1989), with other DR regimens producing greater extensions of lifespan in an N2 genetic background (Guarente and Kenyon, 2000; Houthoofd *et al.,* 2002; Bishop and Guarente, 2007; Greer *et al.,* 2007; Kaeberlein 2006). This method was chosen, as any more severe DR treatments would result in significant loss of wild isolates due to their response to lack of food (crawling out of the petri dish). Given that various DR regimes extend

lifespan via independent and overlapping genetic pathways (Greer and Brunet, 2009), it would therefore be of great interest to determine if variation between genotypes depended on the method of DR.

Most studies in *C. elegans* have been undertaken using one canonical strain, as a reference genotype. The N2 strain was maintained in continuous culture for about 13 years prior to freezing thus subjected to very different conditions to that in the wild (Sterken *et al*., 2015; Petersen *et al.,* 2015). It is therefore likely to be extensively adapted to laboratory conditions (Sterken *et al*., 2015; Rockman, 2012) and the genes affecting longevity and the DR response could have been affected by this. Recently, more thought has been given in *C. elegans* research to comparisons of N2 with natural wild isolates (Andersen *et al.,* 2012; Volkers *et al.,* 2013; Thompson *et al*., 2013). Knowing that genetic variation can significantly influence response to DR a closer look at the freshly derived-wild isolates is therefore needed to obtain a more realistic idea about the effects of DR and to find the natural polymorphic alleles involved in the response to DR. This also highlights the need for studies in general to be carried out in various genetic backgrounds.

CHAPTER THREE: QTL Mapping in Multi-Parent Recombinant Inbred Lines

Abstract

There have been many studies in *C. elegans* that have focused on single laboratory strains and the derived mutants. This is potentially limiting as it does not exploit the wider variation present in the species. Quantitative genetic approaches have also been limited as most analyses have relied on two parental crosses. Although these have been informative, it is unclear how relevant this approach is to understanding natural populations. Given that phenotypic plasticity is determined by genotype-environment interactions, research in multi-parent crosses derived from wild isolates potentially offers a better understanding of allelic interactions and is a great resource to study natural variations. In this work I present extensive analysis of lifespan, dietary restriction, oxidative stress, heat shock and cold shock in a new 4-parent panel of 200 sequenced and genotyped recombinant inbred lines (RILs) without the canonical strain N2. These RILs are good representatives of natural genetic variants. The analyses show there is an extensive variation among the RILs under all phenotypic traits and given their origins this is not lab adaptation. Furthermore, and contrary to expectations, the results indicate that there is no correlation between response to the stresses analysed here and lifespan.

3.1 Introduction

The phenotype of an organism is determined by its genotype (G), by the environment (E) that it experiences and by the interaction between these two factors (the GxE interaction). Given that such responses are also affected by other genetic variants present within the genome, *i.e*. by the genetic background, understanding such responses ideally requires studying multiple environmental stresses in multiple genetic backgrounds. Such responses are debated and partially explained in life history theory, with the assumption that in order for an organism to survive and reproduce it has to adapt its optimized fitness to different stresses and environments (Stearns, 1992).

Individuals of a species have very similar characteristics yet can differ in many ways. It is this difference that is called natural variation. Natural variation and genetic diversity are influenced by several factors, namely: mutation, recombination, selection, genetic drift and demography (Cutter, 2006) as these are the main reasons for populations genetics. Studying natural variation can prove useful for analysing the genetic basis of response to various environmental factors. This can help to understand the effect of a mutation in different environmental conditions and can uncover the response to selection in response to changing environments. Stressful conditions can reveal hidden genetic variability within the individual of the species. For example, *Drosophila melanogaster* kept under high stress conditions, show increased levels of genetic variation in several traits compared to low stress conditions (Swindell and Bouzat, 2006). Similarly, the response of plants such as tobacco (Rizhsky *et al*., 2002), legumes (Pnueli *et al*., 2002) or *Arabidopsis* (Rizhsky *et al*., 2004) to a combination of two different abiotic stresses is unique in comparison to the response of the plants to two stresses individually (reviewed in Mittler, 2006).

Similar to temperature, food availability can also have a profound effect on an organism. One such example is the early development of the nematode *C. elegans*. This worm has two different responses to levels of pheromone and food conditions in order to reach adulthood (Golden and Riddle, 1984). When food conditions are favourable and levels of pheromone are low the worm will develop through the larval stages into adulthood (Riddle and Albert, 1997). However, when the levels of pheromone are high and food becomes depleted, the L1 stage of *C. elegans* larvae makes a decision based on these environmental clues to develop into a specialised L2d (pre-dauer) stage which than develops into a fully arrested L3 stage also called the dauer larvae (Riddle and Albert, 1997). A dauer larva is a dispersal morph that can survive for several months, unlike non-dauer worms which on average live for about 3 weeks in laboratory conditions (Hu, 2007).

One of the major goals in quantitative genetics is to understand the very complex interactions between the genetic factors and the environment (Mackay, 2001). Many effects of genotype by environment interactions produce changes in gene expression. To discover the responsible regulatory loci producing such changes, QTL mapping can be used. Put simply, QTL mapping links two types of data together: phenotypic (trait measurement) and genotypic data (mostly molecular markers), in order to uncover the genetics underlying variation in complex traits such as aging (Lander and Botstein, 1989). The traditional approach for finding QTLs underlying natural phenotypic variation is to analyse large numbers of progeny from two parent crosses in RILs (Brem *et al*. 2002). Since the early days of quantitative genetics, studies in many model organisms ranging from yeast (Cubillos *et al*. 2013), fruit flies (King *et al*. 2012), mice (Durrant *et al*. 2011) and plants (Huang *et al.* 2011) have benefited by expanding on genetic and phenotypic diversity with multi parent RILs. *C. elegans* is notably absent from this list.

Given issues around line construction, a pertinent question is to consider reasons for using QTL mapping rather than a genome wide association study (GWAS) QTL mapping can determine the complexity of the genetic architecture underlying a phenotypic trait and can be effectively used for complex (polygenic) traits such as ageing. GWAS on the other hand relies on having many thousands of SNPs genotyped in a population of unrelated individuals. There are clearly some disadvantages of using QTL method. For example, only the allelic diversity that segregates between the parents of the RIL population can be assayed (Borevitz and Nordborg, 2003). This particular problem, that constrained many earlier QTL studies, can be now partly resolved by introducing multiple parent strains. One such example is four-parent intercross yeast populations (Cubillos *et al*., 2011), another is the multi-parent-advanced-generation-inter-cross in *Arabidopsis thaliana* (MAGIC, Kover *et al.,* 2009), which is constructed from 19 parental strains. Another disadvantage of using QTL mapping is the amount of recombination that occurs during line creation as this limits the mapping resolution (Korte and Farlow, 2013). However, if too much recombination happens it can result in loss of association. GWAS method can overcome these two limitations, as there is no need for recombination and can use many genotypically different individuals, but can introduce other draw-backs. For example, GWAS screens can evaluate the association between each genotyped marker and phenotype of interest in a collection of many unrelated individuals which can produce very detailed map for certain diseases for example. This however, requires high-density marker coverage which may not be available in all organisms as it can be quite costly. In addition, if the trait of interest is present in a very small proportion of the population, then it is not possible to map loci by GWAS. In this instance, QTL mapping can map such trait using bi-parental or multi-parent populations. Another possible approach is to combine GWAS and QTL mapping for precision mapping as demonstrated by Sonah *et al.* (2015). Here, they discovered that all of the GWAS

loci identified for simple traits were successfully validated by 2-parent QTL mapping. Of the three complex traits only the loci for seed weight was validated by both GWAS and QTL (Sonah *et al.,* 2015). While the other two traits (seed oil and protein content) failed to be identified by QTL mapping. This is mainly due to both founder parents having the same alleles at all the oil and protein QTL identified through GWAS. This serves as an example that QTL and GWAS together can form powerful complimentary approach for dissecting complex traits (Sonah *et al.,* 2015).

For the past 30 years, *C. elegans* has been one of the most frequently used model organisms in biological research and *C. elegans* is a valuable model for genetic mapping due to large brood size, very short lifespan, self-fertilizing mode of reproduction and absence of inbreeding depression (Johnson and Hutchinson, 1993). Hermaphroditism allows for rapid inbreeding which is extremely useful for constructing RILs. Traditional QTL mapping of life history phenotypes in *C. elegans* such as fertility, egg size or growth rate have produced different QTLs under different environments, for example analysis at different temperatures identified non-overlapping QTLs for plasticity at age at maturity, body size in maturity and fecundity (Gutteling *et al*., 2007). This indicates that the environment plays an important role in the evolution of quantitative traits and, that to realistically assess QTLs, one must test in multiple environmental conditions.

There is also a need to explore the natural history of *C. elegans*. The usual reference strain, N2, is a laboratory animal that harbours many laboratory-derived variations, particularly noteworthy are the laboratory derived alleles of *npr-1*, *glb-5* and *nath-10* (McGrath *et al*, 2009; Andersen *et al*., 2014; Felix and Duveau, 2012), which appeared as a result of continuous laboratory culturing for approximetly 13 years prior to freezing of this strain (Sterken et al.,

2015). QTL mapping of life history phenotypes in *C. elegans* is therefore very likely to be influenced by these novel mutations. One particularly striking example of this is *npr-1*, where the lab derived allele has been linked to altered behaviour (de Bono and Bargmann 1998), foraging and dispersal (Gloria-Soria and Azevedo 2008), lifespan (Doroszuk papered al., 2009), dauer larvae development in growing populations (Green *et al.,* 2013) and been shown to affect lifetime fecundity, adult body size, and susceptibility to the human pathogen *Staphyloccus aureus* (Andersen *et al*., 2014). Many freshly isolated wild *C. elegans* strains are now available, with many being collected by Marie-Anne Félix (Institute of Biology of the Ecole Normale Superieure, IBENS, FR). Utilizing newly obtained strains for development of recombinant inbred lines is therefore a logical next step in *C. elegans* quantitative genetics.

Here I present an analysis of lifespan under normal conditions (NGM plates and *ad libitum* food) and lifespan under mild dietary restriction (dauer agar plates and restricted food) in a new RIL panel derived from recently isolated wild strains. As discussed in Chapter 1, stress and lifespan are closely related, with many mutations that alter lifespan affecting the stress response and nutrient sensing (Lin *et al*., 1998). Given this, the RILs were also tested for survival after heat shock, cold shock and oxidative stress. These RILs are representative of genotypically distinct groups of *C. elegans* isolates from France and are distinct from both N2 and CB4856. The new panel should therefore be useful in determining the genotype-phenotype relationship in the absence of laboratory derived alleles and be more indicative of subtle allelic effects in wild populations.

3.2 Methods

3.2.1 Construction of RILs

The wild strains used to make the RILs analysed here were freshly isolated by M.A. Félix from two different sites in France. One site was an orchard in Orsay (O), were worms were collected from rotting apples. The second site was a woodland area in Santeuil (S), were worms where collected from rotting hogweed stems. These parental strains were initially genotyped based on the hybridization of genomic *C. elegans* DNA to microarrays (Figure 3.1; see Volkers *et al*., 2013 for details). This resulted in the identification of 6,368 polymorphic genes and showed that the between strain variation is greater than between site variation (Volkers *et al*. 2013). This implies that local genetic diversity reflects strain rather than site-specific signatures. The analysis also revealed four distinct genetic groups (O, S1, S2 and S3) and the parental strains used for the construction of the RILs were chosen to represent each of these genetic groups (Figure 3.1).

After selection of the parental lines, the different lines were crossed for seven generations and then inbred for a further 7-10 generations. This crossing strategy was used to produce 200 genetically distinct lines, and was designed to allow an equal contribution from each of the parental lines and to limit the potential for selection during line construction. Each line was then analysed by RNAseq to investigate a) gene expression and b) to provide line genotype for future QTL mapping.

It is also important to stress that the new multi-parent RILs panel used in this study were derived from freshly collected wild isolates and therefore will not contain any alleles

representing lab adaptation. This fact made culturing of the RILs technically challenging, as many worms crawled off the plates or burrowed into the agar.

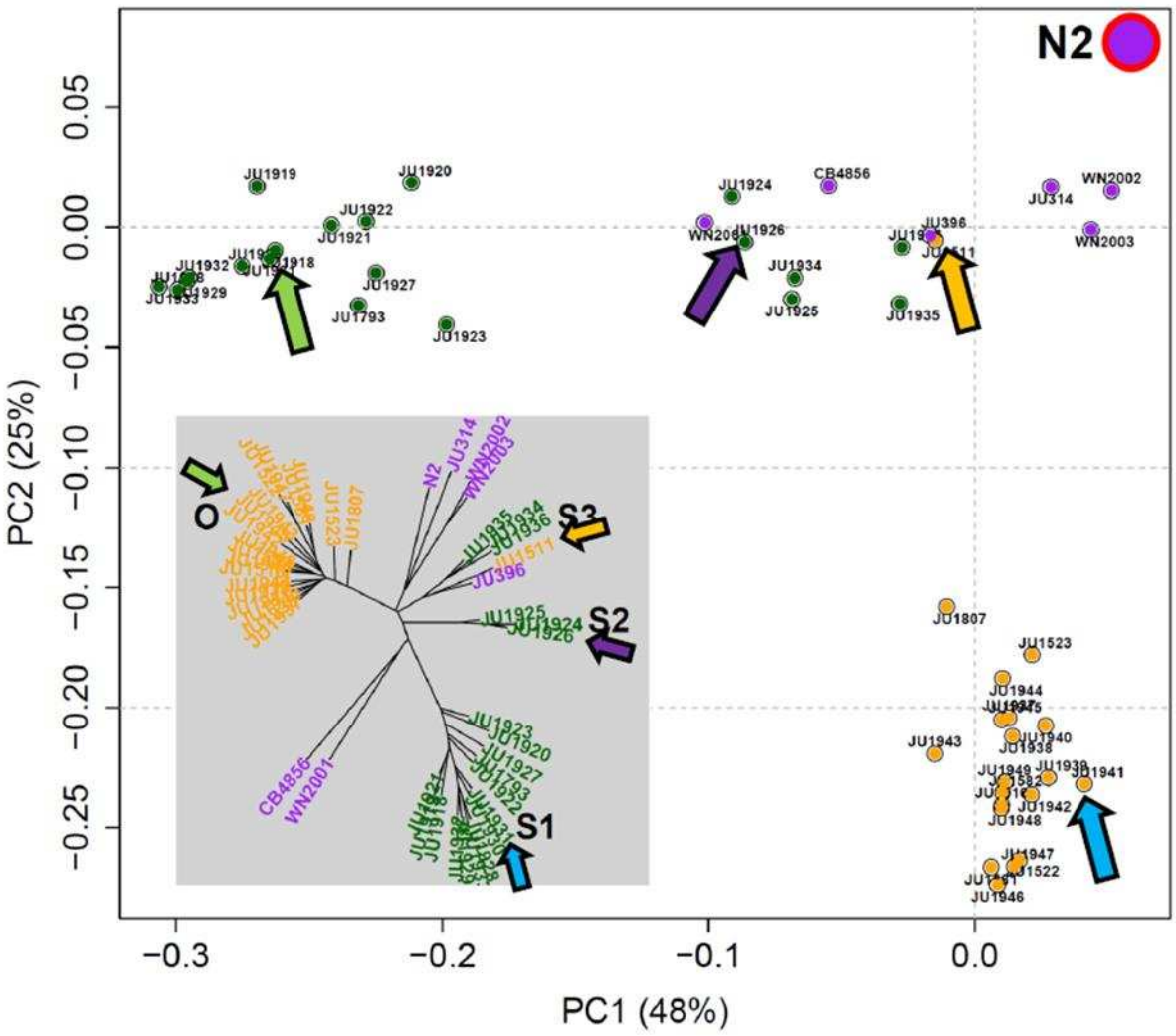


Figure 3.1 Principal component analysis plot (PCA) of parents from genetically separable populations based on genomic DNA analysis. In the new RILs, S1 is represented by JU1941, S2 by JU1926, S3 by JU1511 and O by JU1931. PC1 on the *x*-axis separates the main Santeuil group (green) from all other strains, and PC2 on the *y*-axis separates the Santeuil and out-group strains (purple) from the Orsay strains (yellow). Adapted from Volkers *et al*. (2013).

3.2.2 Lifespan assays

3.2.2.1 Worm lifespan assays

Assays were performed at 20^0C. *C. elegans* populations were synchronized by sodium hypochlorite treatment of gravid adults (Stiernagle, 1995) on NGM without food. The worms were tested for DR in form of bacterial limitation by peptone withdrawal. The method of total withdrawal of peptone from the agarose plates as described by (Hosono *et al*., in 1989) seems to be a relatively mild form of DR (see Chapter 7 for further details of this and a comparison between methods of DR). After 24 hours the plates were seeded with *Escherichia coli* OP50 and the worms were allowed to grow, *en masse*, to the L4 stage. DR was started at the L4 stage, with worms transferred to fresh 35 mm plates at a density of 5 worms per plate and 8 plates per treatment. The plates were then randomised and blind coded such that position in the incubator was not determined by genotype and that worm movement and phenotyping was done without knowledge of genotype. For lifespan under DR conditions, the L4 worms were moved to fresh 35 mm NGM petri plates without peptone (dauer plates), again with 5 worms per plate and 8 plates per treatment. Each RIL within an experimental block was therefore tested under both conditions, with a total of 40 worms per treatment per RIL. The movement of the L4 worms to fresh plates was counted as day one for all the lifespan measurements. All worms were moved every day to fresh plates for the first four days and then every other day to eliminate confounding progeny and, for the DR treatment, to maintain food availability. Worms were scored daily as dead if they did not respond to a gentle touch with a platinum pick, worms that crawled of the plates or died of bagging phenotype were censored out of the experiment. In total, the RILs were assayed in six blocks with 35-48 randomly selected RILs in each block, and with some RILs present in multiple blocks. RILs were not included in any

analysis if less than three worms were observed per lifespan per genotype. This cut-off point was decided on as many RILs did escape the plates. As well as the RILs, N2 was also used in lifespan assays. 5 -fluorodeoxyuridine (FUdR), which is commonly used to prevent reproduction by *C. elegans* hermaphrodites in lifespan and ageing experiments, was not used in this study as it is known to alter lifespan of some genotypes (Anderson *et al*., 2015) and has been known to induce resistance to thermal and osmotic stress (Angeli *et al*., 2013). Given the potential of this to confound results of this work, *i.e.* by identifying variation between lines in response to FUdR rather than lifespan variation, FUdR was not used in these assays.

3.2.2.2 Statistical analysis

Was carried out by one-way ANOVA. The results were used to calculate heritability of the lifespan traits. RIL lifespan under each condition was then tested for correlation. All of these analyses were done in Minitab® Statistical Software (Mintab Ltd., Coventry). To investigate the role of data variance in the effect of dietary restriction on lifespan, a model of lifespan data was developed in R (version 3.1.3, R Foundation for Statistical Computing, Vienna, Austria, (URL http://www.R-project.org/)).

4.2.3 Heat stress resistance

To identify a suitable heat stress treatment that would be long enough to kill some, but not all worms, I carried out several preliminary trials to establish the best method for screening the RILs and N2. After discussion with collaborators the conditions of Rodriguez *et al*. (2012) were used as a starting point. Worms were cultured at 15 ^{0}C prior to the heat shock assays. Worms were allowed to grow *en masse* into adulthood then synchronised by sodium hypochlorite

treatment of gravid adults (Stiernagle, 1995) and then the eggs were collected. After 24 hours, the plates were seeded with *E. coli* and the worms were allowed to grow *en masse* into L4/young adult's stage. At this stage, worms were transferred to fresh plates ten worms per plate with five replicates for each RIL and parental strains. The plates were than randomised and blind coded. Worms were then placed at 35^0C for ten hours. After this ten-hour treatment, worms were allowed to rest at 15^0C for 48 hours before scoring for survival. After 48 hours, worms that did not respond to a gentle prod with the worm pick were scored as dead. Worms that crawled of the plates or died of bagging were censored from the experiment. The proportion of worms was then calculated and used for analysis.

4.2.4 Oxidative stress resistance

To test the oxidative stress resistance of the RILs, I measured survival of worms treated with hydrogen peroxide (H_2O_2). As with the heat stress assays, I carried out several trials with serially diluted H_2O_2 in order to define an LD_{80}, rather than using LD_{50}, in N2. A higher dosage was used as it was expected that the RILs, being constructed from recent wild isolates, would have the potential to withstand higher levels of oxidative stress compare to the laboratory kept N2. The LD_{80} was determined by treating N2 with a serial dilution from 10% to 0.1% of H_2O_2 for 24 hours and defined a test concentration of 4% H_2O_2. For the main experiment, worms were maintained at 20°C, synchronized as described above and grown *en masse* to the L4 stage. At L4 the worms were washed of the plates with M9 and 10-30 individuals transferred to 96 well plates in a total volume of 48 μl, with three replicates for all the RILs and N2. The plates were then transferred to a WMicrotracker-One™ to measure activity of the worms in the wells (Phylum Tech, Sunchales, Argentina) in a constant temperature room at 20 °C and activity over

30 minutes determined. After this step, 2 µl of 4 % H_2O_2 solution was added to all wells, giving a final volume of 50 µl, except for the control. Worms were then incubated for 24 hours at 20°C. After 24 hours, the activity of the worms was measured again using the WMicrotracker-One™. The WMicrotracker-One™ records movement as photo-beam interruptions (bins) within wells of 96-well plates. Given this, the effect of oxidative stress was calculated as: (reading after 24 hours - reading before treatment)/(reading before treatment) = activity score. An activity score of -1 therefore represents all worms being dead at the end of the treatment, and an activity score of 0 indicates that a similar level of movement is recorded after treatment. This score also can generate values activity score above zero, indicating that some more movement was recorded after the H_2O_2treatment.

4.2.5 Cold stress resistance

To measure cold stress resistance, synchronized populations were obtained as described above and cultured on NGM plates at 20^0C until the L4/ young adult's stage. At this stage, the plates were transferred directly to 4^0C ±0.5^0C (Savory *et al*., 2011) for 14 days. At the end of this cold treatment, plates were moved to 20^0C and worms left for 24 hours to recover. This prolonged recovery is important as there is significant mortality over the first few hours after transfer from cold stress in *C. elegans* (Robinson and Powell, 2016). After 24 hours, the numbers of living and of dead worms was counted and used to determine the proportion of worms surviving. Worms that did not responded to prodding with the worm pick were scored as dead and worms that did respond were scored as alive (as is common in determining dead or alive state of the worm). All RILs were scored within one assay, with one plate per RIL and

the assay was repeated three times with mean survival calculated as the average of the three replicates.

Statistical analysis for the various stresses were carried out by one-way ANOVA. The results were used to calculate heritability of the lifespan traits. RIL survival under each condition was then tested for correlation (table 1). All of these analyses were done in Minitab® Statistical Software (Mintab Ltd., Coventry).

4.3 Results

4.3.1 Lifespan and DR

Analysis of the lifespans of the RILs under both *ad libitum* and restricted food conditions shows that there is extensive and continuous variation between the RILs under both conditions and no obvious step-like patterns, suggesting a polygenic control (Figure 3.2a and b, all RILs and average lifespans can be find in Appendix A). Some of the RILs show high variability within the lines (a large standard error). This is partially due to the small sample sizes for some RILs due to high levels of bagging observed, a consequence of the lack of FUdR, and the number of worms that crawled off the plates. The lines also show extensive variation in the difference between lifespan under the two conditions, with a large number of the lines showing decreased mean lifespan under conditions of mild dietary restriction (figure 3.2c). Here, one can observe that although the majority of the RILs show the expected response, *i.e.* DR prolonging lifespan, there are some lines (about 1/3) that show a shortened lifespan under the mild DR treatment. This analysis also uncovered a potential outlier (line Y16), which was extremely long lived in both of the lifespan assays (top right corner of Figure 3.3).

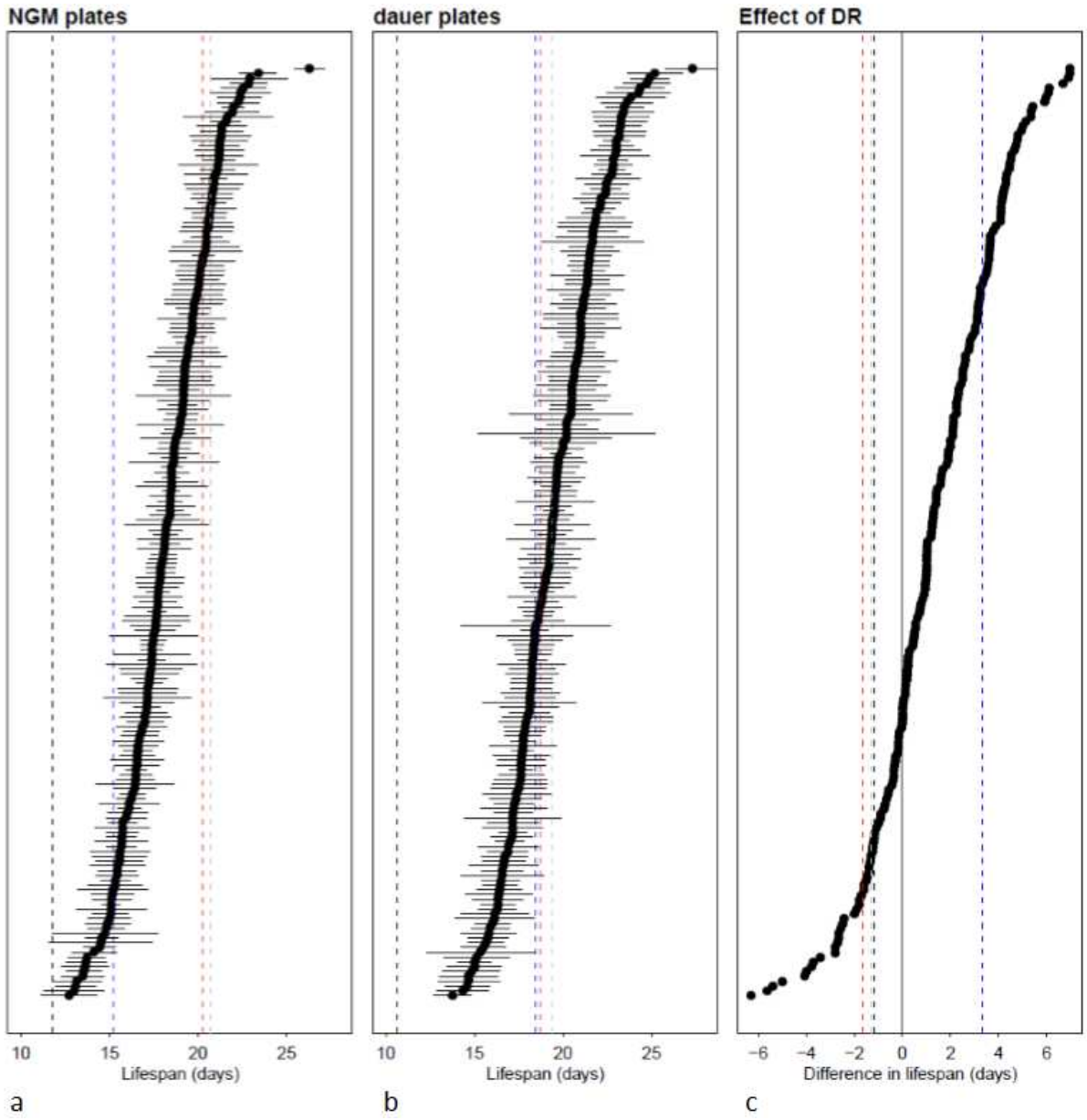


Figure 3.2 Lifespan under normal conditions and dietary restriction in the RILs. Shown are the ranked mean lifespans of the RILs (±1 standard error) under normal (NGM plates a) and DR (dauer plates b) conditions. The third plot represents the effect of DR (c) on lifespan as calculated by lifespan under DR - Lifespan under normal conditions. RILs are sorted by trait value within each plot. The vertical lines represent mean lifespan in the parental strains, the black line representing JU1511, the grey line representing JU1941, the red line representing JU1931 and the blue line representing JU1926.

When the parental lines are compared, we can observe that one parent, JU1511, is extremely short lived in both treatments and that the lifespan of this line is slightly decreased by DR treatment. Of the other parental strains, both JU1931 and JU1941 were also shorter lived under DR conditions, with only JU1926 showing an extension of lifespan. N2 controls in these assays lived on average for 19 days (normal) and 20 days (DR) respectively. Comparison of the lifespan between the different treatments indicates that lifespan is related, but there is a large degree of variability between the RILs (Figure 3.3). This also indicates that, on average, DR extends lifespan.

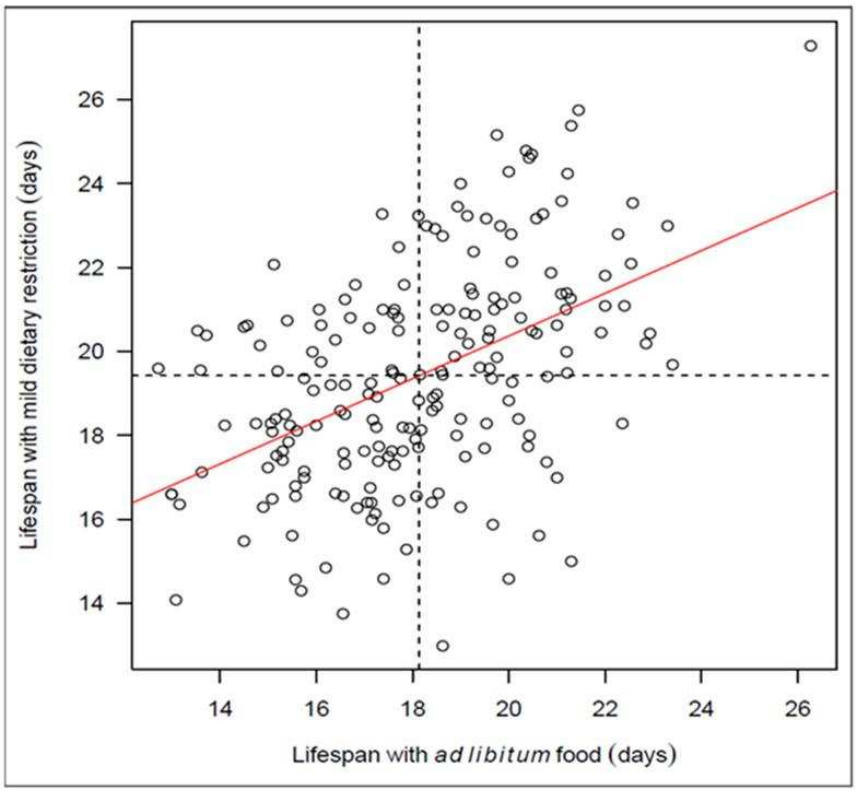


Figure 3.3 Lifespan on NGM and dauer plates. *Ad libitium* lifespan is on the *x*-axis and lifespan under DR condition is on the *y*-axis. Average lifespan of all RILs for *ad libitum* and DR conditions is indicated by the vertical and horizontal dotted lines. The line of best fit is positive, indicating a positive relationship between the two lifespans. The higher mean for lifespan under DR conditions indicates that the overall effect of DR is to extend lifespan.

The lifespan analyses were run in six blocks in total. To test for a block effect, the lifespans of RILs that were duplicated in different experimental blocks were plotted against each other (Figure 3.4). This indicates a positive correlation between lifespan in the different blocks (Pearson correlation of 0.335, p = 0.037) and hence that despite variability between blocks, responses are consistent. Estimates of heritability calculated from one-way ANOVA indicate a marginally higher heritability for the normal lifespan (0.25) than for the lifespan under mild DR (0.23).

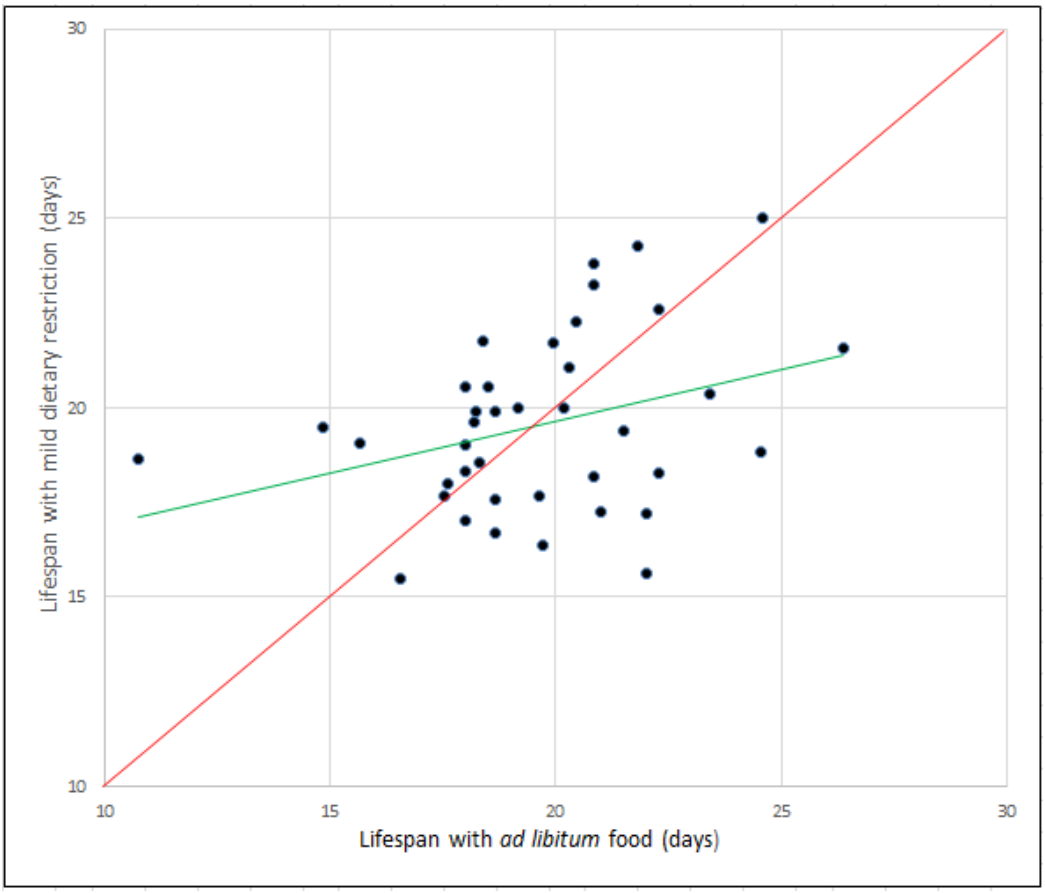


Figure 3.4 Block effect in RILs. Average lifespans from RILs appearing in multiple blocks were plotted against each other to test for block effect. The red line represents a 1:1 and the green line represents the line of best fit.

To test to what extent the small number of recorded lifespans per line may influence the apparent effect of DR, simulated lifespan data were generated assuming that all simulated lines had the same mean lifespan and the same within line variance. These were taken at the grand mean of the RIL lifespan and the variance of one set of N2 control. It was assumed that the overall effect of DR was a lifespan extension of one day (based on the overall average lifespan for *ad libitum* and DR, Figure 3.5, see appendix B for the R script).

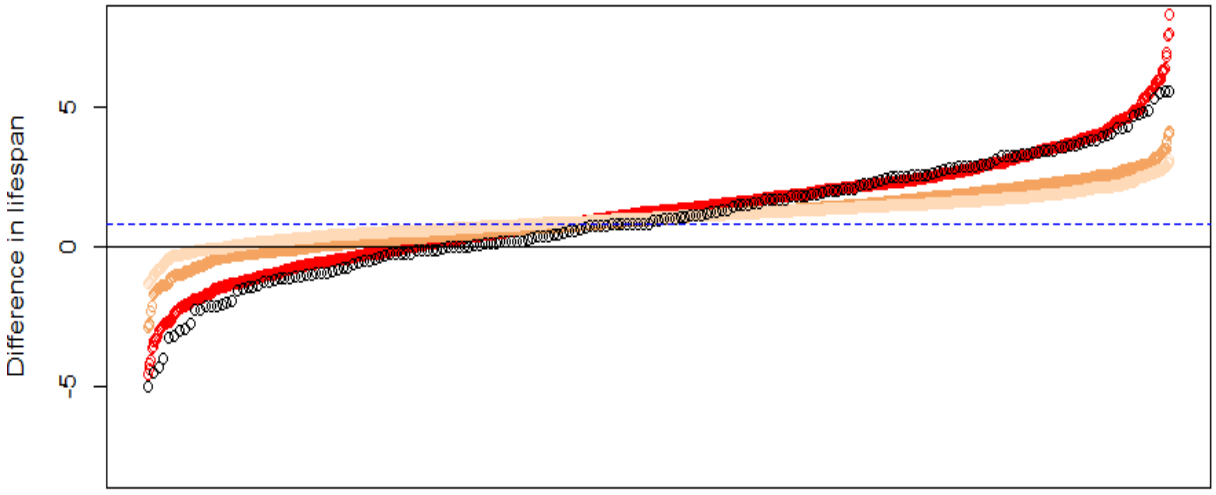


Figure 3.5 Simulation of lifespan differences. represents differences in lifespan for 1000 lines assuming an 18.1 day mean lifespan, a 1.3 day increase in average lifespan from DR, a normal distribution of individual lifespan within a line, and 5 (red), 10 (brown) and 20 (pink) worms per treatment for each line. Overlaid on this are the observed difference for the RILs (black circles).

This shows that with a normal distribution of lifespan and a genuine positive effect of DR on lifespan, that a number of lines would be expected to show a negative effect of DR. This is particularly apparent when only 5 lifespans per treatment are simulated, although it is also apparent with larger sample sizes. When plotted alongside the difference between DR and *ad libitum* lifespan from figure 3.2c, the real data curve closely follows the 5 data input on the positive side of the figure (life prolonging effect of DR) but tails of on the negative side (DR

shortening of lifespan). As the real data had, on average, 10 worms per line (range 3-22), the fact that the curves do not match implies that the observed RIL data is showing a real biological response, *i.e.* there are RILs in which DR is not having an effect within which the effect of DR is to reduce lifespan.

4.3.2 Survival in response to stress

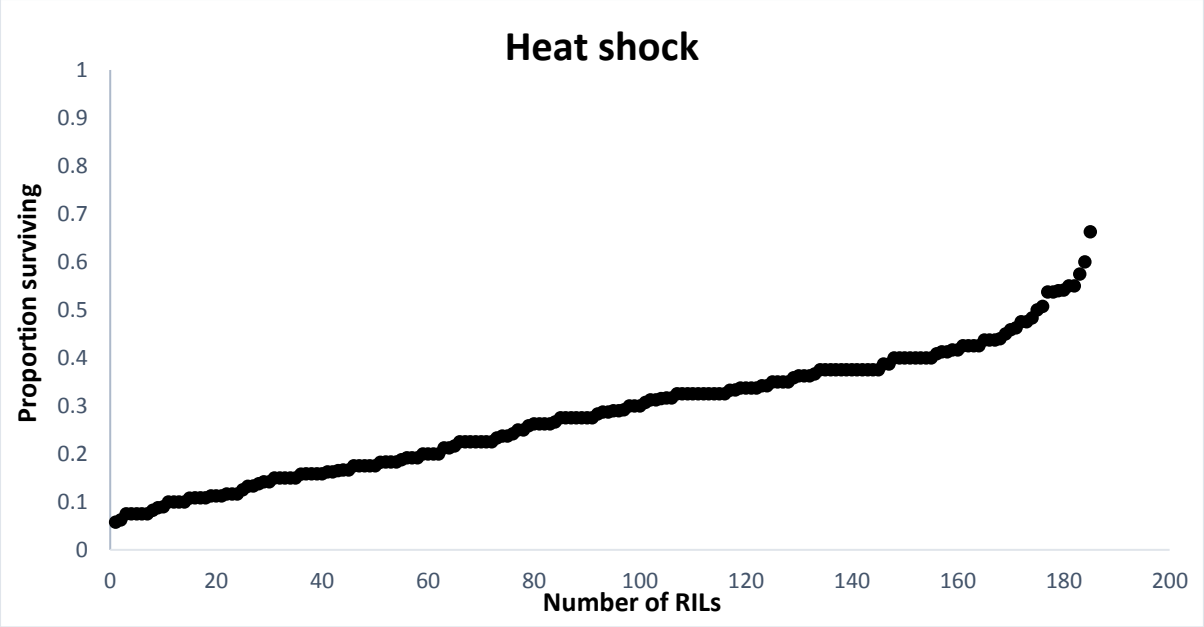
As with the lifespan assays, the RILs were highly variable for all three stresses assayed (Figure 3.6). Here we can again observe several outliers. For example, line Z25 could best withstand the cold treatment (97% of worms surviving), whilst line ZY7 seems to be most resistant to heat shock and YZ25 is extremely active 24 hours after hydrogen peroxide treatment. Interestingly, and despite the prediction that lifespan and stress resistance should be related, correlation of the various stresses and the lifespan traits indicates that only the lifespans under various conditions shows any correlation (table 3.1).

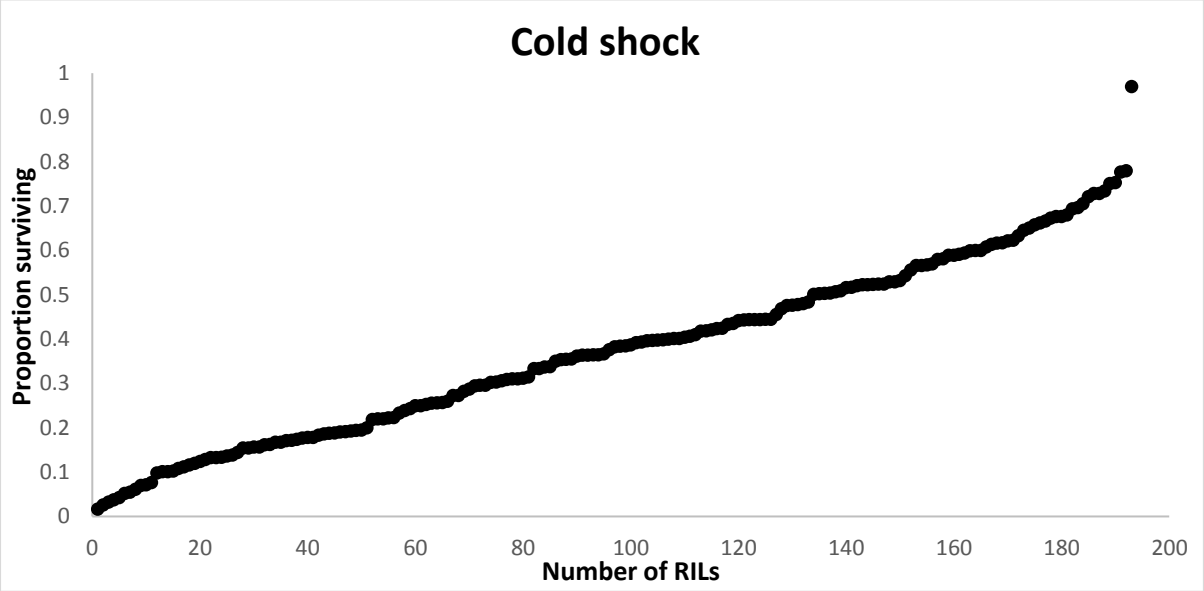
		LS	DR	HS	CS
DR	1	0.461			
	2	<0.001			
HS	1	0.027	0.018		
	2	0.72	0.811		
CS	1	-0.057	-0.058	0.108	
	2	0.432	0.421	0.145	
OS	1	0.096	0.084	0.104	0.021
	2	0.271	0.316	0.243	0.807

Table 3.1 Relationships between measured traits. Shown are the pairwise correlations between lifespan under normal conditions (LS), lifespan under DR (DR), heat stress resistance (HS), cold stress resistance (CS) and oxidative stress resistance (OS). Number 1 represents the Pearson correlation and number 2 represents the *p* value.

All of the stress assays were tested on approximately 180 RILs (Figure 3.6). The reason for this is that some of the lines (approx. 20) exhibited extremely high rates of maternal hatching, which in turn made it difficult to use such lines in the assays. Heritability for heat shock was calculated from one-way ANOVAs and was very similar to those of lifespan (22.5%). Heritability for cold shock and oxidative stress, however, was much higher (68% and 58%, respectively).

The analysis for cold shock resistance uncovered an interesting response. Here, the RILs responded in an almost Mendelian fashion (trait is controlled by a single locus in an inheritance pattern). This could be a possible explanation for high heritability score. Given the genetic variation of the RILs, one can postulate that the response to cold shock is governed by a small number of genes, therefore lines have high heritability for this trait. Testing for the resistance to oxidative stress by hydrogen peroxide also resulted in unusual findings. The Microtracker recorded activity in individual wells before the hydrogen peroxide treatment and after 24 hours in the treatment were collected and converted to proportions, giving scores as follows (1 all dead to 0 all alive). However, scores above zero were also recorded, indicating that some RILs were more active after the


Heat shock
Proportion surviving
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
20
40
60
80
100
120
140
160
180
200
Number of RILs


Cold shock
Proportion surviving
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
20
40
60
80
100
120
140
160
180
200
Number of RILs

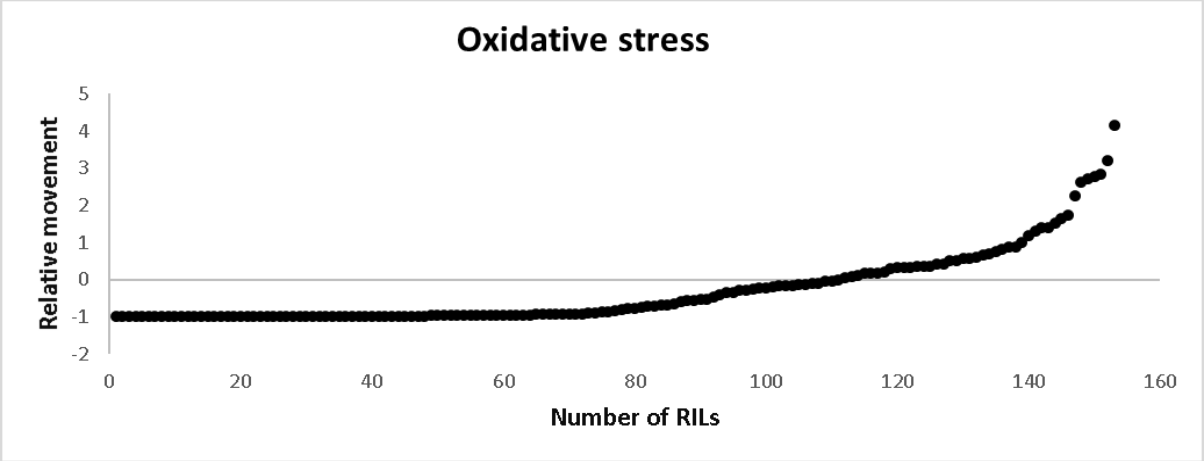

Oxidative stress
Relative movement
5
4
3
2
1
0
-1
-2
0
20
40
60
80
100
120
140
160
Number of RILs

Figure 3.6 Variation between RILs in response to heat shock, cold shock and oxidative stress. Shown are the proportion curves of worms surviving after heat stress and cold stress (value of -0 indicating all worms dead and value of -1 indicating all worms alive and the proportion curves of worms after oxidative stress (value of -1 indicating that all worms were dead). Observation of these RILs uncovered rather surprising response to hydrogen peroxide shock treatment, making some RILs more active after 24 hours in treatment.

4.4 Discussion

Previous studies in *C. elegans* have investigated the organisms' response to various environmental factors such as temperature (Harvey and Viney, 2007; Vinuela *et al.* 2011), various bacteria (Sinha et al. 2012), pH (Grishkevich *et al.* 2012), chemicals (Gidalevitz *et al.* 2013) and osmotic pressure (Rohlifing *et al.* 2010). These studies are important for understanding the biology of complex organisms. Most of the studies, however, have been carried out on just a few genetic backgrounds. As stated previously in this chapter, low allelic diversity can seriously underestimate the complex genetic architecture of polygenic traits. The newly constructed multi-parent RILs were used in this study with the hope of addressing these issues.

I have analysed the novel multi-parent RILs for response to various environmental factors. Different treatments revealed variance in lifespan under normal conditions and under mild DR conditions. Here, the RIL analysis shows that there is extensive variation in lifespan under both conditions (figure 3.2). These data indicate extensive transgressive segregation, i. e. the formation of extreme phenotypes, the result of each parent containing alleles that increase

lifespan and others that decrease lifespan, or of negative epistasis (i. e. declining of fitness) between alleles from different parents. The responses of the RILs and the parental lines to DR also seems to uncover that even mild DR affects lifespan in a genotype specific manner. This has not been observed in *C. elegans* before work on this thesis started (as reviewed in Chapter 3), but has now been seen in introgression lines and wild isolates (Chapter 3/Stastna *et al*., 2015) and in a new panel of RILs (figure 3.2c). A trivial explanation might be that the effect is an artefact of many small sample sized genotypes and it is apparent that such a pattern can be generated in this way (figure 3.5). This is, however, unlikely to fully explain my results, given that extremely small sample sizes are required in the simulations and that the real data show an over-representation of lines with a negative effect of DR on lifespan.

There is also significant variation among these RILs in response to other environmental stresses. This indicates that the recent wild isolates are likely to possess powerful protective responses that enhance their survival in the wild – these may have been lost or degraded in N2 due to adaptation to laboratory conditions. This makes sense given that wild populations within the natural environment are often exposed to longer periods of less extreme environmental changes and therefore possess the ability to withstand such conditions. In addition, the response to these stressors differs among the phenotypes (Li *et al.,* 2006). Again, this may be explained by taking into consideration *C. elegans'* natural habitats where the worms have to deal with many environmental stresses (Felix and Duveau, 2012; Petersen *et al*., 2014). It has been proposed that laboratory induced stress is not equal to stress encountered in the natural environment, for example in Walker *et al*. (2000) it was observed that populations of long-lived *age-1* mutants were outcompeted by wild type under cyclic starving conditions. It has also been suggested that permissive laboratory conditions remove

the resource constraint that enforces the trade-offs between lifespan and various types of stress resistance such as the immune system as it is costly to maintain (Reznick and Ghalambor, 2005). Keeping animals in standard laboratory conditions over a prolonged period of time can therefore potentially lead to a lack of selection against the high stress resistance. Keeping this in mind, all of the stress assays were performed on the worms within a few generations of thawing from -80^{0}C.

The global diversity of *C. elegans* is comparatively low and the species displays little geographical structure in contrast to local genetic diversity (Barriere and Felix, 2005; Cutter, 2006; Volkers *et al*., 2012; Andersen *et al*., 2012). In most of these studies the diversity was measured as genetic diversity with very little interest in phenotypic variation of the freshly derived wild isolates (Volkers *et al*., 2012). Therefore, creating and phenotyping of multi-parent RILs derived from wild isolates was the next logical step. This panel is a good representation of natural genetic variants and can be used as a starting step for unravelling functional analyses of allelic variants which are likely to play a key role in the control of many complex traits. For this reason, QTL mapping of the RILs and candidate gene approach is the next step of this work.

CHAPTER FOUR: QTL Mapping

QTL mapping reported in this Chapter was performed by L.B. Snoek (Wageningen University) as the RIL genotype data is currently unpublished and is therefore embargoed.

Abstract

Over the last few decades the importance of *C. elegans* as a genetic model has been steadily rising. Most of the earlier studies have analysed many, mainly monogenic, traits whilst using strains that are now known to be not entirely suitable. Using approaches such as quantitative trait loci (QTL) can reveal the complex mechanism underlying life history traits. Currently the biggest challenge is the choice of strains that allow for accurate mapping. The novel panel of 4-parent recombinant inbred lines (RILs) derived from freshly isolated wild strains offers a new opportunity to study multi-allele interactions that are likely to occur in the wild. Bin mapping in this panel revealed 20 potential QTLs for lifespan, dietary restriction, the effects of dietary restriction, heat shock, cold shock and oxidative stress. There was a great variation among the RILs for all the traits that define these QTLs. Importantly, some QTLs cannot be explained by the known regulators of ageing in *C. elegans*, *i.e.* QTL mapping in the strains has identified variation in previously unknown regulators of lifespan. Overall, this novel panel therefore represents an exciting new resource in QTL mapping that has been notably missing in *C. elegans*.

4.1 Introduction

Over the last few decades, the field of genetics has generated vast amounts of data. This has been driven by the development of a wide array of methods to search for genes underlying phenotypic variation. The early studies, especially, have been extremely useful for initially characterising and subsequently identifying causative genes for a huge range of traits. For example, Sydney Brenner characterised hundreds of *C. elegans* genes by EMS mutation (Brenner, 1974). Later, development of analysis methods such as RNA interference (Fire *et al.*, 1998), targeted gene knockouts (Frøkjær-Jensen *et al*., 2010) or microarrays (Blumenthal *et al*., 2002) allowed for collation of more data on the function of many genes. Most of these early studies have however focussed on monogenic traits. Useful as they were, concentrating on a single gene or small groups of genes, however, ignores the many intermediate levels that are involved in the final phenotype of an organism and this approach can be therefore seen as an oversimplification of nature.

Using approaches such as QTL analysis coupled with the use of RILs can help to unveil the genetic mechanisms underlying complex quantitative phenotypic variation. QTLs are regions within genomes (also known as loci) that contain genes associated with a particular variation in a phenotype (the quantitative trait). QTLs can be mapped by identifying which molecular markers (SNPs or AFLPs) correlate with an observed trait (Collard *et al*., 2005). This strategy for identifying the genetic basis of complex traits has been somewhat underutilized (Gaertner and Phillips, 2010). This may be possibly because in the face of a proliferation of traditional forward and reverse genetic approaches, which can produce much larger effects than are attributable to QTLs, thus mapping of complex traits such as longevity may appear to be an inefficient approach to define the genetic underpinnings of longevity. Despite these draw-backs, a

quantitative method allows for an unbiased approach for detection of natural longevity variants for complex traits as well as the estimation of effect size and effect direction of multiple alleles across the genome. Although early quantitative genetic studies utilised inappropriate strains (with high mutation rate) such as crosses of BO Bergerac for example in early studies with N2/BO RILs (Johnson and Wood, 1982; Johnson, 1987; Brooke and Johnson, 1991; Ebert *et a*l., 1993), or BO/RC301 RILs (Ayyadevara et al., 2001) and BO/CL2a RILs (Ayyadevara et al., 2003), later studies used more appropriate strains such as Bristol and Hawaii N2/CB4856 (Anderson *et al*., 2007; Li *et al*., 2006; Gutteling *et al*., 2007; McGrath *et al.,* 2009; Doroszuk *et al*., 2009; Rockman *et al.,* 2010) or Bristol and Californian strain N2/DR1350 (Viney *et al.*, 2003; Harvey *et al.,* 2008; Harvey *et al.*, 2009; Harvey, 2009). Overall, these studies have identified many QTLs, for example these studies have uncovered natural variation in sensitivity to volatiles (Van Swinderen *et* al., 1997) and oxygen levels (Persson *et al*., 2009). QTLs affecting variations in complex traits have also been mapped, these include longevity (Doroszuk *et al*., 2009), phenotypic plasticity to temperature (Gutteling *et al.,* 2007a), influence of temperature on various life-history traits (Gutteling *et al*., 2007b), locomotion (Harvey, 2009) and gene expression (Palopoli *et al.,* 2008; Li *et al*., 2010, Rockman *et al*., 2010).

The first successful QTL mapping of genome regions affecting longevity found survival QTLs in liquid media (Ebert *et al.*, 1993, Ebert *et al*., 1996, Shook *et al*., 1996). Similar but not identical QTL locations were then obtained by the Johnson group who used a more conventional and versatile strategy with N2/BO RILs on solid media. This confirmed the presence of longevity QTLs on chromosomes I, lll and X (Shook and Johnson, 1999). Early work of Ayyadevara *et al*. (2001) uncovered 12 potential QTLs for lifespan on all chromosomes in populations derived from an RC301/BO strains and N2/BO strains. Using CLA2/BO crosses yielded eight significant

lifespan QTLs on chromosomes I, lll, lV, V and X (Ayyadevara *et al*., 2003). These results are partially supported by Doroszuk *et al.* (2009) study using introgression lines of N2/CB4856 strains. Here, out of in total six detected lifespan QTLs three of those on chromosomes I, lll and X were close to the earlier findings (Doroszuk *et al*., 2009). It is, however, rather difficult trying to compare all of the previously detected QTLs as various studies used different lines, maps and markers. For instance, there is no guarantee that a co-localising QTL identified in RILs produced from different sets of parental lines results from variation in the same gene(s). Similarly, much of the early work is potentially attributable to transposon-induced mutations as BO has lost the ability to silence transposons and is essentially mutationally degraded, making the Bergerac strain very unstable (Riddle *et al*., 1996).

Currently, one of the greatest challenges of quantitative genetics is the choice of the strains that would allow for efficient and accurate mapping of phenotypic traits. Also there is a need for detection of QTLs with small effects with the aim to detect causal genes. As previously mentioned, QTL mapping in *C. elegans* can and will benefit from the new multiple parent lines derived from wild isolates as these lines bear closer resemblance to natural populations in many ways. As well as increasing variation, these lines introduce the possibility of more than two independent alleles at a locus and provide space for potential epistatic interactions (Huang *et al*., 2012). In this chapter, I will analyse the results of QTL mapping and look for candidate genes for lifespan, dietary restriction, the effects of dietary restriction, heat shock, cold shock and oxidative stress.

4.2 Methods

Phenotypic data from traits assayed in the four parent RILs, as described in detail in Chapter 4, were mapped in J. Kammenga's Nematology laboratory (Wageningen University, Holland). The

threshold for all of the traits was a LOD score of 3, denoting a 0.05 genome-wide threshold. LOD score is a measure of the strength of evidence for the presence of a QTL in particular location. In this study LOD (γ) = $\log_{10}$ likelihood ratio comparing the hypothesis of a QTL position γ versus that of no QTL. This resulted in the detection of 20 potential QTLs for the various traits (figures 4.1-4.6 and table 4.1). The criteria to define QTL regions was based on the LOD scores –of a QTL at a particular location. If the LOD score appeared over the 3 LOD threshold, therefore being significant, all LOD scores above 2 for the given genotype were than included in mapping of the overall QTL position. For example, QTL on chromosome l for lifespan under DR has peak LOD score of 2.99, with 14,428,949 position in the genome for JU1511 strain. When taken in consideration all LOD scores above 2 for JU1511 the size of the QTL is 683,908 bp spanning from 14,174,121 – 14,858,029.

Once the QTL regions were defined for all the traits, candidate genes were sought after. Some QTL regions were too big to consider all individual genes, for example the QTL for lifespan under normal conditions on the X chromosome spans a 8,662,743 bp region from 5,997,980 – 14,660,723 (table 4.1). For all of the QTL regions the WormMine tool (WS253, Intermine data mining platform for *C. elegans* and related nematodes) was used to find how many and which genes underlie the individual QTL regions (table 4.1). As well as this broad observation, for the genomic regions that were sufficiently small to search through, the genome browser at WormBase (Version WS254) have been searched for individual genes and their functions.

Mutations that postponed senescence were first discovered in worms and as one of the consequences have led to discovery of the evolutionary conserved Insulin/Iinsulin-like growth factor signalling (ILS) pathway (Kenyon *et al.*, 1993, see figure 1.1 in Chapter 1). In *C. elegans* this pathway is central to both growth and metabolism (Tullet *et al.,* 2008). Due to the central

role of this pathway and other important signalling pathways such as the mechanistic target of rapamycin (mTOR) in ageing in *C. elegans,* I searched WormBase (Version WS254) for genes that are located within these pathways (see Appendix C for a list of these genes and their predicted functions). This list of known ageing-related genes was compared to the QTL regions defined in this work. Given the close relationship between lifespan and stress (previously discussed in Chapters 1 and 4), I would expect to see at least some of these genes underlying the QTLs. Further evidence for candidate genes was sought based on the genome sequence of the parental lines and on previous analyses of gene expression (data from Wormbase).

4.3 Results

4.3.1 Lifespan under normal conditions

Bin mapping of average lifespan under normal condition resulted in five QTLs (Figure 4.1, Table 4.1). The QTL plots are coded by the four parental genotypes and the potential combination of 2 parents. Each dot represents an individual single nucleotide polymorphism (SNP). These plots determine whether there is an association of the regions of the genome (QTLs) with observed traits, with dots above the threshold line indicating that variation at this point in the genome is associated with variation in the trait. The first lifespan QTL on chromosome l (lsq1, LOD 3.28, size 294,857 bp, Table 4.1) can be assigned to alleles shared by the JU1511 and JU1926 parents (Figure 4.1). There are no known genes from conserved lifespan-related signalling pathways in lsq1 (Table 4.2). Consideration of wider data for the genes underlying this QTL does however identify some potential candidates.

Trait	Chrom	Name	Position	Peak position	TOP LOD score	Number of genes
LS	I	lsq1	3214050 - 3508907	3319533	3.28	60
	I	lsq2	4639748 - 8048021	7013267	3.27	1063
	II	lsq3	14281875 - 14366334	14363904	3.31	17
	III	lsq4	2843344 - 9943924	3356724	4.88	2252
	X	lsq5	5997980 - 14660723	11425905	4.8	3124
DR	I	drq1	4671128 - 7774527	7485288	3.2	966
	I	drq2	14174121 - 14858029	14428949	2.99	156
	IV	drq3	1093503 - 2184563	1325343	3.54	234
	X	drq4	4022118 - 12986819	5237857	5.24	3840
DR effect	III	efq1	3228492 - 5255539	3420394	2.96	635
HS	X	hsq1	2606330 - 4714196	3404162	3.43	763
	X	hsq2	8538113 - 10577289	8538113	3.69	680
	X	hsq3	12450150 - 12748621	12450150	3.59	82
	X	hsq4	12847478 - 14176212	13326502	3.02	482
CS	I	csq1	3558266 - 4131703	3996252	3.27	136
	I	chq2	11536185 - 15058135	12653112	4.42	1009
	III	csq3	2340285 - 7276973	4346553	9.65	1449
	III	csq4	2556178 - 8006105	7149007	3.21	620
OS	III	osq1	2404894 - 7316336	6422312	3.54	1451
	X	osq2	10243642 - 17711068	16974981	7.16	2462

Table 4.1 Summary of all detected QTL for all traits. LS represent lifespan under normal conditions, DR represent lifespan under DR conditions. DR effect is difference that DR conditions have on lifespan and was calculated as (DR-LS). HS stands for heat shock, CS stands for cold shock and OS stands for oxidative stress.

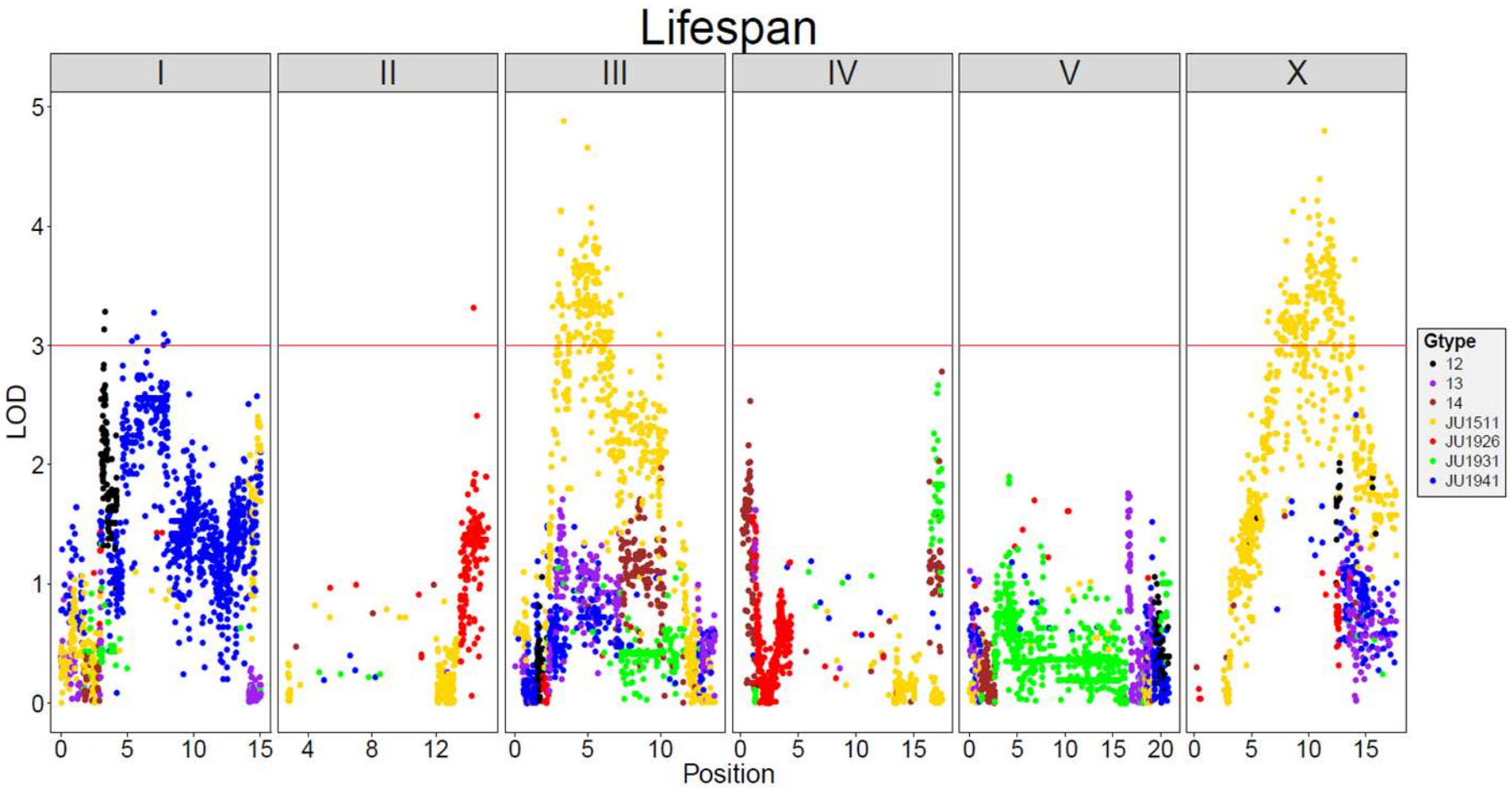


Figure 4.1 QTL mapping for normal lifespan

The six chromosomes are shown and the red line represents a genome-wide threshold. Dots above the threshold indicates that variation at this point in the genome is associated with variation in lifespan, and indicate the presence of a QTL. These plots are coded by four parental genotypes and by combination of two parents (Gtype). Each dot represent an individual SNP.

For example, out of the 60 genes found in lsq1, Y44E3A.6 and Y54E10A.17 are both regulated by *age-1* (a central component of ILS pathway), and W05F2.7 is regulated by *sir-2.1* (a regulator of ageing via Insulin/insulin-like growth factor DAF-16). Another gene in this region is *tag-63* which is regulated by *skn-1* (again, *skn-1* is part of ILS and MSPK pathway). The second lifespan QTL (lsq2, LOD 3.27, size 3,408,273 bp, Table 4.2) can be assigned to allele(s) from JU1941 (Figure 4.1). This QTL spans over nearly three and half million base pairs and harbours several genes known to be involved in ageing or stress response (*aap-1, smg-1, let-363, unc-14 and iftb-1*).

QTL for lifespan located on chromosome II (lsq3, LOD 3.31, size 84,459, Table 4.2) is the smallest detected QTL from all traits. Lsq3 can be assigned to allele(s) from JU1926 parent (Figure 4.1) and there are only with 17 genes underlying this QTL of which five have been previously identified in ageing or stress response (*ddl-3, ctl-1, ctl-2, ctl-3 lin-7*, Table 4.2, Table 4.4). Chromosome III has one QTL region with the highest LOD score for lifespan (lsq4, LOD 4.88, size 7,100,580 bp, Table 4.2) which can be assigned to JU1511. Lsq4 spans about a third of the chromosome and harbours many genes including *daf-2,* which is central to ILS and *akk-1* which is associated with mTOR signalling. Detailed analysis of all polymorphisms between the parental strains identifies ten polymorphisms in the *daf-2* region with no non-synonymous changes in JU1511 (Appendix D). This does not exclude the possibility of regulatory polymorphisms, but does indicate that there are peptide changes in *daf-2* in JU1511. The lsq4 region also contains Y53G8AL.2, which is involved in determination of adult lifespan. Lifespan QTL chromosome X (lsq5, LOD 4.8, size 8,662,743 bp, Table 4.2) can be also assigned to parental strain JU1511 (Figure 4.1). There are several genes underlying lsq5 with can be linked

to various signalling pathways (*sem-5, akt-2, ist-1, ftt-2, daf-9, daf-12, daf-3, vhl-1, mdl-1, lin-2* and *jkk-1*). Underlying the peak of this QTL is *aco-1*, this gene is required for normal lifespan and L4-to-adult growth rates.

4.3.2 Lifespan under DR and Effect of DR

Mapping of lifespan under mild DR resulted in a total of four QTLs on chromosomes I, IV and X (Figure 4.2, Table 4.1). The first QTL on chromosome I (drq1, LOD 3.2, size 3,103,399 bp, Table 4.2) can be assigned to parental strain JU1941 and the region contains 966 genes of which at least five are known to influence lifespan or feature in the stress response (*aap-1, smg-1, let-363, unc-14* and C10H11.8). Underlying the peak of drq1 is T28F4.4, a gene that from microarray studies appears to be regulated by *daf-12*, *set-2* and *slr-2*. The second QTL on chromosome I (drq2, LOD 2.99, size 683,908 bp, Table 4.2) can be assigned to JU 1511 and the identified region contains 156 genes. The strongest candidate in the region is the *catp-1* gene, which likely functions together with the *let-60*/Ras and *daf-2*/ILS to regulate L2 larval developmental timing and dauer formation.

The lifespan under DR QTL on chromosome IV (drq3, LOD 3.54, size 1,325,343 bp, Table 4.2) is the only one detected on this chromosome. No know genes involved in the ageing-related signalling pathways were found within drq3, however, F53H1.3, which is predicted to be involved in determination of adult lifespan is located in close proximity to the drq3 peak. The lifespan under DR QTL on chromosome X (drq4, LOD 5.24, size 8,964,701 bp, Table 4.2) is partially co-locating with lsq5. This is mostly due to the fact that both of X chromosome QTLs span over eight million base pairs – almost half of the whole X chromosome. As expected given the size of the region, many genes that play role in the ageing-related signalling pathways were found in this region (*ist-1, ftt-2, daf-9, daf-12, daf-3, cst-1, jkk-1, vhl-1, mdl-1, lin-2, sem-5* and

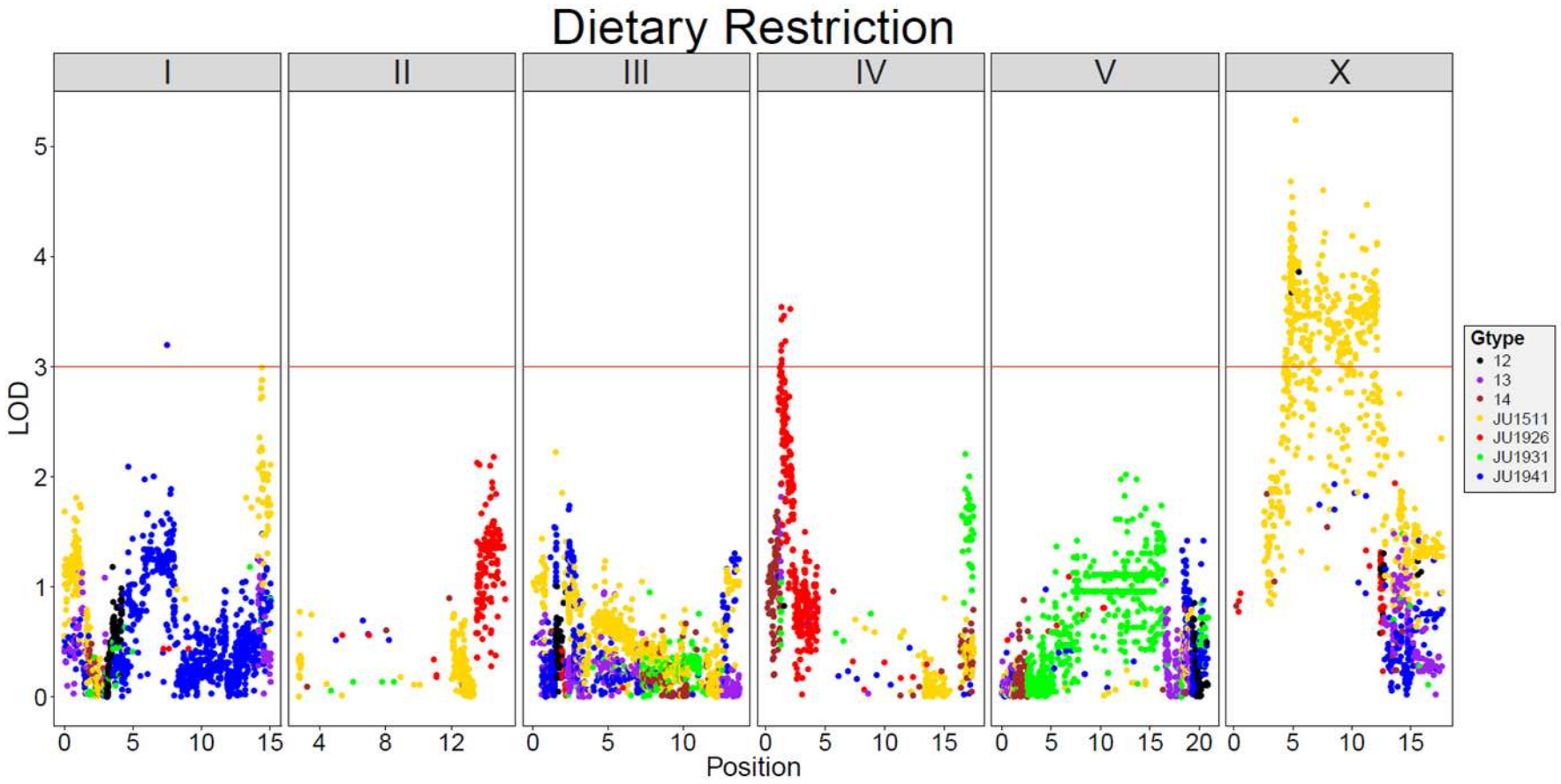


Figure 4.2 QTL mapping for lifespan under DR

The six chromosomes are shown and the red line represents a genome-wide threshold. Dots above the threshold indicates that variation at this point in the genome is associated with variation in lifespan, and indicate the presence of a QTL. These plots are coded by four parental genotypes and by combination of two parents (Gtype). Each dot represent an individual SNP.

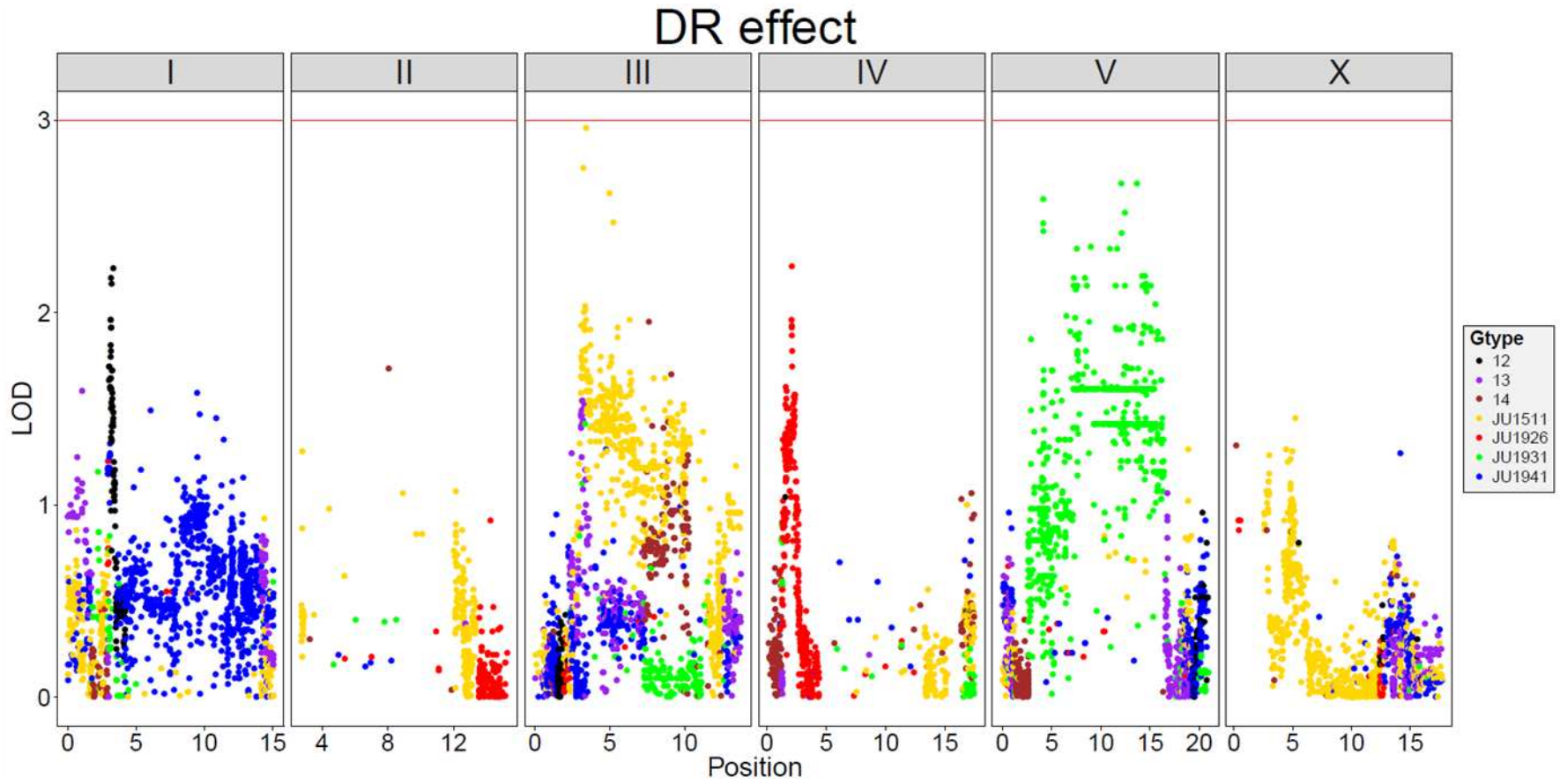


Figure 4.3 QTL mapping for effect of dietary restriction.

The six chromosomes are shown and the red line represents a genome-wide threshold. Dots above the threshold indicates that variation at this point in the genome is associated with variation in lifespan, and indicate the presence of a QTL. These plots are coded by four parental genotypes and by combination of two parents (Gtype). Each dot represent an individual SNP.

jkk-1). To observe the effects of DR on lifespan, DR - lifespan QTL was also plotted (figure 4.3, table 4.1). There was only one suggestive QTL detected on chromosome lll (efq1, LOD 2.96, size 2,027,047 bp, Table 4.2), This potential QTL co-locates with lifespan QTL lsq4 with peak LOD scores located at similar position within the genome and both attributed to the JU1511 parent, but this is not significant at a genome wide level.

Trait	Chr	Size bp	Peak	Genes previously shown to effect ageing or the stress response that were found here
LS	I	294,857	3,319,533	Y44E3A.6
	I	3,408,273	7,013,267	*aap-1, smg-1, let-363, unc-14, iftb-1*
	II	84,459	14,363,904	*ddl-3, ctl-1, ctl-2, ctl-3 lin-7*
	III	7,100,580	3,356,724	*daf-2, rheb-1, akk-1, daf-7, daf-4, mpk-1, tir-1, med-2*
	X	8,662,743	11,425,905	*sem-5, akt-2, ist-1, ftt-2, daf-9, daf-12, daf-3, vhl-1, mdl-1, lin-2, jkk-1, mek-1, sek-1, med-1*
DR	I	3,103,399	7,485,288	*aap-1, smg-1, let-363, unc-14*, C10H11.8
	I	683,908	14,428,949	
	IV	1,091,060	1,325,343	
	X	8,964,701	5,237,857	*ist-1, ftt-2, daf-9, daf-12, daf-3, cst-1, jkk-1, vhl-1, mdl-1, lin-2, sem-5, jkk-1, mek-1, sek-1, med-1*
LS-DR	lll	2,027,047	3,420,394	*mpk-1, tir-1*
HS	X	2,227,799	3,404,162	*cst-1*
	X	3,329,476	8,538,113	
	X	298,471	12,450,150	*med-1*
	X	1,328,734	13,326,502	*akt-2*
CS	I	573,437	3,996,252	
	I	3,521,950	12,653,112	*hsf-1, fkb-2, gsk-3, rps-6*
	lll	4,936,688	4,346,553	*daf-2, daf-4, mpk-1, tir-1, med-2*
	lll	5,449,927	7,149,007	*daf-2, daf-4, mpk-1, tir-1, med-2*
OS	lll	4,911,442	6,422,312	*daf-2, daf-4, mpk-1, tir-1, med-2*
	X	7,467,426	16,974,981	*akt-2, sgk-1, sod-3, ftt-2, aak-2, daf-12, vhl-1,lin-2,med-1*

Table 4.2 All genes detected in various QTL regions. All of these genes have been previously identified in ageing or stress response pathways. Genes are underlying previously identified QTLs.

4.3.3 Stress response

Bin mapping of heat shock resulted in four potential QTLs all on chromosome X (Figure 4.4, Table 4.1). The first QTL (hsq1, LOD 3.43, size 2,227,799 bp, Table 4.2) can be assigned to parental strain JU1511 and this region contains the gene *cst-1*, which functions downstream of DAF-16 and appears to play a role in the responses to oxidative stress and determination of adult lifespan. The second QTL on the X chromosome (hdq2, LOD 3.69, size 3,329,476 bp, Table 4.2) can be assigned to single parent strain JU1941.

Genes underlying peak of the hsq2 are *nspc-1/4/5/6,* these genes are regulated by *clk-1*. In *C. elegans*, CLK-1 activity is required for normal physiological rates of growth, development, behavior, and aging. The third QTL for heat shock response (hsq3, LOD 3.59, size 298,471 bp, Table 4.2) can be assigned to parental strains JU1511 and JU1926. Although there are no known ageing or stress response genes found underlying hsq3, T18D3.9, a gene that is regulated by *daf-12* can be found under the peak of the QTL. This is interesting because *daf-12* is known to affect gonad-dependent adult longevity together with DAF-16. The last of the detected heat stress QTLs (hsq4, LOD 3.2, size 1,328,734 bp, Table 4.2) can be assigned to alleles from JU1941. Underlying this QTL is *akt-2*, which is part of ILS pathway and acts upstream of *daf-16*, *sir-2.2* and *sir-2.3*, and F46G10.2. There are four genes in *C. elegans* that are similar to SIR-2 (silent information regulator 2, family of NAD^+ dependent enzymes) or sirtuins for short and play an important role in molecular mechanisms of ageing (Tissenbaum and Guarente, 2001). RNA sequencing studies indicate that F46G10.2 is regulated by *daf-12, eat-2, daf-2, hcf-1* and *sir-2.1.*

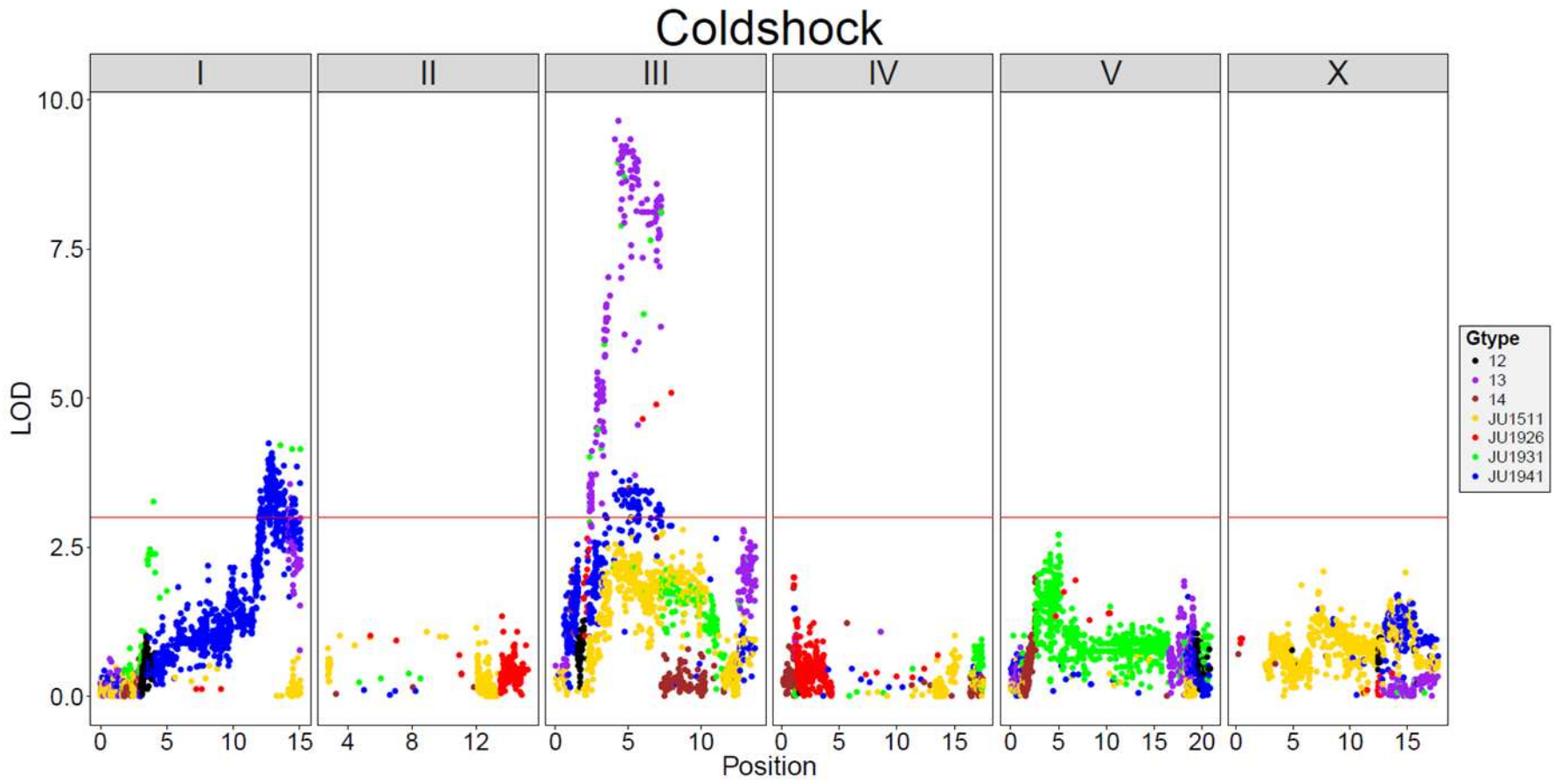


Figure 4.4 QTL mapping for response to heat shock.

The six chromosomes are shown and the red line represents a genome-wide threshold. Dots above the threshold indicates that variation at this point in the genome is associated with variation in lifespan, and indicate the presence of a QTL. These plots are coded by four parental genotypes and by combination of two parents (Gtype). Each dot represent an individual SNP.

Four potential QTLs were found for cold shock on chromosomes I and III (Figure 4.5, Table 4.1). The first QTL on chromosome I (csq1, LOD 3.27, size 573,437 bp, Table 4.2) can be assigned to JU1931 parental strain. No genes from the ageing-related signalling pathways were detected in the csq1 region, but the region does contain F28H1.1 which is regulated by *daf-16*, *sir-2.1* and *isp-1*. The second QTL on chromosome I (csq2, LOD 4.42, size 3521950 bp, Table 4.2) can be assigned to JU1941 parental strain. There are several genes important for ageing and stress response underlying csq2 (*hsf-1, fkb-2, gsk-3* and *rps-6*). Underlying the peak of this QTL is *mcrs-1*, evidence based on RNA sequencing, and microarray studies indicate that *mcrs-1* is regulated by *daf-12*, *sir-2.1* and *tdp-1*. Out of all QTL detected in the 4-parental RILs, the one found on chromosome III had the highest LOD score (csq3, LOD 9.65, size 4,936,688 bp, Table 4.2). Csq3 can be assigned to parental strains JU1511 and JU1941 and co-locates with lsq4, efq1, osq1 and csq4. Genes underlying csq3 are *daf-2* and *daf-4.* At peak of csq3 is the gene F56F3.4, an ortholog of human ZFAND5 a gene involved in lipid storage. The last cold shock QTL on chromosome III (csq4, LOD 3.21, size 5,449,927 bp, Table 4.2) can be assigned to JU1941. Genes underlying csq4 are *daf-2* and *daf-4*. Under peak of csq4 is R02F2.8. Microarray studies indicate that R02F2.8 is regulated by *daf-16* and *tdp-1*. The gene *tdp-1* is involved in the oxidative stress response and in proteotoxicity, exhibits a dose-dependent effect on lifespan, and is required for the long lifespan and oxidative stress resistance of *daf-2* mutants. Also located under this peak is *osg-1*, which also plays a role in aging, and appears to be regulated as part of the oxidative stress response.

Mapping of oxidative stress detected two potential QTLs on chromosomes III and X (Figure 4.6, Table 4.1). QTL on chromosome III (osq1, LOD 3.54, size 4,911,442 bp, Table 4.2) co-locates with csq3 and can be attributed to same JU1941 parental strain. Underlying this QTL are *daf-2*

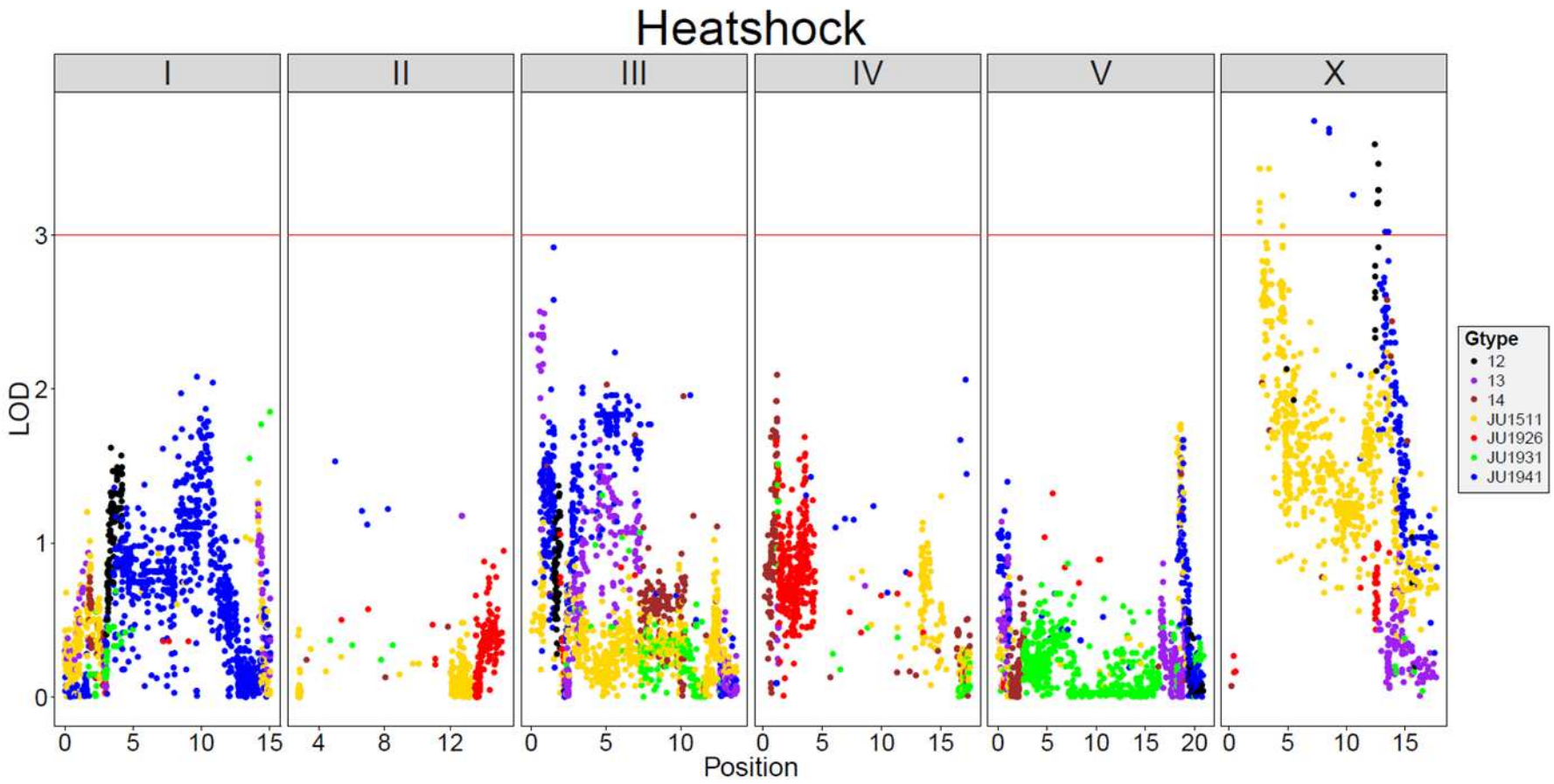


Figure 4.5 QTL mapping for response to cold shock.

The six chromosomes are shown and the red line represents a genome-wide threshold. Dots above the threshold indicates that variation at this point in the genome is associated with variation in lifespan, and indicate the presence of a QTL. These plots are coded by four parental genotypes and by combination of two parents (Gtype). Each dot represents individual SNP.

and *daf-4* and at the peak is *atgl-1*, a gene involved in lipid storage and reproduction. The QTL on chromosome X (osq2, LOD 7.16, size 7,467,426 bp, Table 4.2) can also be assigned also to JU1941 parent and this region contains many genes related to lifespan and the stress response (*akt-2, sgk-1, sod-3, ftt-2, aak-2, daf-12* and *vhl-1*). Underlying peak LOD score are F52G3.5 (regulated by *sir-2.1* and *pmk-1*) and F52G3.1. Tiling array and microarray studies indicate that F52G3.1 is regulated by *daf-12*, *ain-2*, and *fbf-1*).

Comparison of QTLs for the different traits indicates that most of the co-localization appears to be on chromosome lll, but this is most likely due to the size of the detected QTLs. For example, lsq4, scq4, csq4, and osq1 all spanning almost 5 million base pairs each, which is about third of the whole chromosome lll. The QTLs efq1 and lsq4 also collocate on lll, have very similar peak LOD scores (3,420,394 and 3,356,724 bp respectively) and can be attributed to same parental strain JU1511.

In total six of all of the detected QTLs for LS, DR, DR effect and Heatshock can be attributed to JU1511. During various assays, this parental strain, comparing to other parental strains, was the most distinguishable one due to its small size and very short lifespan. The QTL lsq3 on chromosome ll was the smallest detected (84,459 bp), and hence allowed a more detailed analysis of underlying genes. Using WormMine (WS253), I found 17 genes underlying this QTL, of which five have been previously identified in ageing or stress response (Table 4.3). Analysis of sequence polymorphisms between the 4 parents within this region identified a total of 150 polymorphisms. Of these, 98 distinguished JU1926 from the other parental strains and 15 polymorphisms were present within *ctl-1, ctl-2, ctl-3, ddl-3* and *lin-7.* This identified three non-synonymous substitutions, two in *lin-7* and one in *ctl-2* (Table 4.4). Although *lin-7* has been shown to be regulated in an age-related manner (Vinuela *et al.,* 2012), the role of the gene in

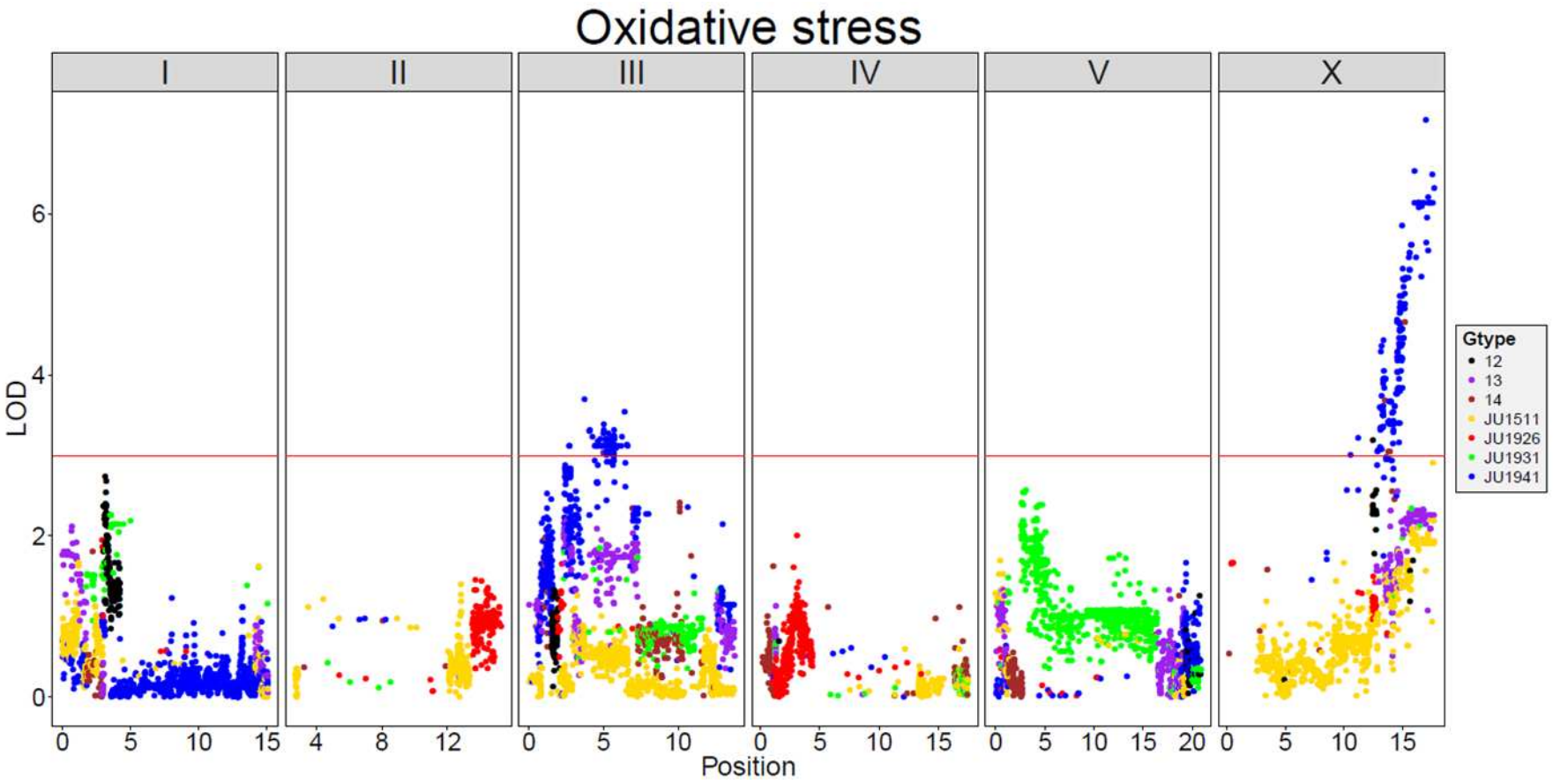


Figure 4.6 QTL mapping for response to oxidative stress.

The six chromosomes are shown and the red line represents a genome-wide threshold. Dots above the threshold indicates that variation at this point in the genome is associated with variation in lifespan, and indicate the presence of a QTL. These plots are coded by four parental genotypes and by combination of two parents (Gtype). Each dot represents individual SNP.

specifying vulval development make it unlikely that it is a candidate for the lifespan effects seen here. Given this, *ctl-2* represents a strong candidate for the causative gene for this QTL.

Gene	Overview
Y54G11A.3	**Y54G11A.3** is predicted to have ATP binding activity and nucleic acid binding activity, based on protein domain information.
srh-41	***srh-41*** Microarray studies indicate that ***srh-41*** is regulated by Resveratrol.
Y54G11A.4	Microarray, tiling array, and RNA sequencing studies indicate that **Y54G11A.4** is regulated by *zfp-1*, *lin-35*, *hpl-2*, *wdr-23*, *blmp-1*, *ubc-9*, *skn-1*, *glp-1*, *sir-2.1*, *ash-2*, *spg-7*, *tbx-2*, and *isp-1*.
Y54G11A.7	**Y54G11A.7** is involved in embryo development.
ctl-2	***ctl-2*** encodes one of three *C. elegans* catalases; *ctl-2* activity is required for normal lifespan as well as for the extended lifespan seen in *daf-2* mutant animals.
ctl-1	***ctl-1*** encodes one of three *C. elegans* catalases; *ctl-1* activity contributes to the extended lifespan seen in *daf-2* mutant animals; in addition, *ctl-1* expression is negatively regulated by DAF-2-mediated insulin signalling.
ctl-3	***ctl-3*** encodes one of three *C. elegans* catalases; CTL-3 is predicted to function as an antioxidant enzyme that protects cells from reactive oxygen species.
Y54G11A.14	Microarray and RNA sequencing studies indicate that **Y54G11A.14** is regulated by *daf-12*, *cyc-1*, *lin-15B*, *tatn-1*, *pgl-1*, *pgl-3*, *glh-4*, and *glh-1*.
ddl-3	***ddl-3*** is an ortholog of human TTC19 (tetratricopeptide repeat domain 19); *ddl-3* is involved in determination of adult lifespan.
lin-7	***lin-7*** expression in vulval epithelial cells is detected only upon overexpression via heat shock.
Y54G11A.9	**Y54G11A.9** is involved in determination of adult lifespan and lipid storage;
Y54G11A.17	**Y54G11A.17** is involved in receptor-mediated endocytosis.
Y54G11A.11	**Y54G11A.11** is involved in embryo development.
agl-1	***agl-1*** is involved in the breakdown of the complex sugar, glycogen.
pfs-2	***pfs-2*** is involved in locomotion, the molting cycle and nematode larval development.
R06A4.2	**R06A4.2** is regulated by Rotenone, Progesterone, Testosterone, Resveratrol, Chlorpyrifos, Diazinon, Humic substances, and Paraquat.
imb-2	***imb-2*** is involved in RNA interference, body morphogenesis, embryo development, locomotion, nematode larval development, receptor-mediated and endocytosis

Table 4.3 Genes under lying lsq3 on Chromosome II. The position for the QTL is 14281875 – 14366334 with peak at 14363904, spanning 84459 bp. This information was obtained from WormBase (Version WS254, data mining platform for *C. elegans* and related nematodes).

CHR	POS	REF	ALT	QUAL	GENE	POSITION		VARIATION
II	14,301,272	A	G	720	ctl-2	II:14,299,553..14,302,083	JU1926 I, others T	WBVar01252910
II	14,303,737	T	C	31.8085	ctl-1	II:14,303,612..14,306,329	exonic	low confidence
II	14,307,970	T	C	29.7836	ctl-3	II:14,307,854..14,310,779	exonic	low confidence
II	14,341,029	GTTTTTTTTTT	GTTTTTTTTTTT	181	ddl-3	II:14,340,068..14,344,454	intron	WBVar01406961
II	14,341,750	T	G	561	ddl-3	II:14,340,068..14,344,454	intron	Same as WBVar01252923
II	14,342,432	C	G	720	ddl-3	II:14,340,068..14,344,454	intron	WBVar00014613
II	14,342,982	A	T	720	ddl-3	II:14,340,068..14,344,454	intron	WBVar01252925
II	14,346,327	G	A	360	ddl-3	II:14,340,068..14,344,454	5' UTR	WBVar01563160
II	14,347,284	T	C	720	lin-7	II:14,346,877..14,349,406	JU1926 T, others I	WBVar00556754
II	14,347,729	GTTTTTTTT	GTTTTTTTTT	299	lin-7	II:14,346,877..14,349,406	intron	WBVar01393688
II	14,347,750	A	G	720	lin-7	II:14,346,877..14,349,406	intron	WBVar00014658
II	14,347,880	A	T	720	lin-7	II:14,346,877..14,349,406	intron	WBVar00014663
II	14,348,090	GTTTTTTTTT	GTTTTTTTTTT	240	lin-7	II:14,346,877..14,349,406	intron	WBVar01393699
II	14,349,393	C	G	720	lin-7	II:14,346,877..14,349,406	JU1926 G, others A	WBVar00014673
II	14,349,403	C	T	720	lin-7	II:14,346,877..14,349,406	5' UTR	WBVar00014678

Table 4. 4 The analysis of sequence polymorphisms for lsq3.

Parental strain JU1926 was distinguished from the other parental strains and 15 polymorphisms were detected within *ctl-1, ctl-2, ctl-3, ddl-3* and *lin-7. ctl-2* represents a strong candidate for the causative gene for this QTL.

4.4 Discussion

This study has detected 20 QTLs for lifespan, dietary restriction, and the effects of dietary restriction, heat shock, cold shock and oxidative stress in 4-parent RILs. There were five QTLs for lifespan under normal conditions on chromosomes I, II, III and X. This study confirms four previously detected QTLs for lifespan on chromosomes I, II, III, and X (Ayyadevara *et a*l., 2001; Shook *et al*., 1999; Ayyadevara *et al*., 2003 and Doroszuk *et al*., 2009), although these studies were based on mapping populations derived from different parental strains. There was no detectable QTL on chromosome IV for lifespan, despite it being previously reported by other studies (Ebert *et al*., 1993; Ayyadevara *et al*., 2001; Ayyadevara *et al*., 2003; Doroszuk *et al*., 2009). This is likely to be due to the use of different parental strains without N2 that will harbour different allelic variants.

I have detected multiple genes from various signalling pathways underlying lifespan QTLs (Table 4.2). These include some of the important main components of the Insulin/insulin-like signalling pathway (*daf-2, ist-1, aap-1, akt-2, sgk-1* and *hsf-1*). Mutations that reduce the activity of the insulin-like receptor DAF-2 can significantly prolong lifespan in *C. elegans* (Kenyon et al., 1993). To investigate if the major detected QTL for lifespan lsq4 is due to differences between the parental strains, I searched for polymorphisms between the parental strains. The results show that although there were ten polymorphic variations between JU1511 and other parental strains these were all no non-synonymous changes in *daf-2* and, as there is also no expression difference in the gene within the RILs (L.B. Snoek, pers com.), this gene is unlikely to be responsible for lsq4 (Appendix D, Table D1).

Comparing normal lifespan to lifespan under DR conditions uncovered one QTL that partially co-locates on chromosome X. Both of these QTLs harbour many genes from various ageing-

related signalling pathways (Table 4.2) and span over eight million base pairs, however with very different peaks (Table 4.1). Mapping for lifespan under DR yielded three QTLs on chromosomes I, IV and X. QTL at the beginning of chromosome IV is co-locating with previously detected lifespan QTL form Doroszuk *et al*. (2009) study. There were no known genes underlying this QTL indicating that not all of the QTLs can be explained by the genes known to be part of cell signalling. There were several genes that were predicted to be involved in determination of adult lifespan for example F53H1.3 and Y77E11A.7 as well as several genes that were regulated by genes from ILS signalling pathway such as *age-1, skn-1* and *daf-16* (K03H6.2, Y41D4B.17 and Y41D4B.15). Mapping for effect of DR on lifespan resulted in one QTL (efq1), this QTL co-locates with efq1 and both share very similar LOD peak scores (Table 4.1). This possibly means that both QTLs are due to the same loci.

QTLs for heatshock were located on chromosomes III and X. These results are partially supported by Shmookler-Rreis *et al*. (2006) who detected a heat shock QTL on chromosome III, but differ from the findings Rodriguez *et al*. (2012) where QTLs underlying heatshock were located on chromosome II and IV. One possible explanation can be difference in laboratory protocols; in previous study the worms were heat shocked for 4 hours at 35 ^{0}C whereas in my study the heat shock was at 35^{0}C for 10 hours. This difference is due to my preliminary study where 80% of mortality was achieved after 10 hours at 35^{0}C . Also in Rodriguez *et al*. (2012) the heatshock was used as a tool of hormesis, where most of the worms survived and their lifespan and fecundity were than measured. In my study the exposure to heat shock was much longer and worms were sorted into dead or alive after 48 hours. This is a good example of difficulties with comparing data from different QTL studies. Not only are there different strains that harbour many laboratory-derived mutations (for review see Sterken *et al.,* 2015) and some

especially early crosses with Bergerac BO were highly unstable (Riddle *et al.,* 1996), there are great differences in protocols.

When comparing results for cold shock and oxidative stress, it appears that there is an almost identical QTL that can be assigned to allele(s) from the JU1941 parent on chromosome III for both traits (csq4 and osq1) as well as a much larger QTL that is shared between parental strains JU1941 and JU1926. This could possibly mean that there is a JU1941 allele that affects both traits and an allele in both JU1941 and JU1926 that only affects cold stress. This can possibly suggest pleiotropic or closely linked QTL stress response under both cold shock and oxidative stress conditions. In previous studies, QTLs affecting oxidative stress were detected on chromosome lV (Vertino *et al.,* 2011 and Andersen *et al.,* 2015). As both studies used different strains CL2a/BO in Vertino *et al.* (2011) and N2/CB in Andersen *et al*. (2015), the difference in the outcomes can be potentially assigned to choice of the freshly-collected natural wild isolates rather than using laboratory strains. Given that poikilotherms response to chronic cold stress is by increasing Δ^9 desaturase gene expression (Tiku *et al*., 1996), I was surprised that the three *C. elegans* Δ^9 desaturase genes (*fat-5/6/7*, located on chromosome V) were not detected by QTL mapping. This is an interesting outcome and will be further discussed in Chapter 6.

I found several co-locating QTLs for various traits for lifespan and stress response in novel 4-parent RILs. This suggests pleiotropic or closely linked QTLs associated with lifespan and stress response. Mapping for these traits in some QTLs have been too broad to achieve definitive implication of underlying genes. On the other hand, there were several QTLs that allowed for closer inspection of candidate genes and their polymorphisms. The results, where some of the QTLs cannot be explained by genes underlying various signalling pathways, are especially interesting as this implies that there are potentially many genes without known functions that

are crucial to ageing and stress response not being previously detected. Overall, this study shows that the novel multi-patent panel provides a versatile resource toward easier and efficient fine mapping and functional analyses of loci and genes underlying complex traits which can accelerate the discovery of natural polymorphisms underlying complex traits and will lead to a better understanding of the mechanisms behind the observed phenotypic variation in *C. elegans*.

CHAPTER FIVE: Natural variation in the cold stress resistance in *Caenorhabditis elegans*

Abstract

Animals in the wild have evolved multiple survival strategies in order to cope with environmental changes. Temperature regulation, especially, is generally a critical requirement for survival and proliferation, with the ability to survive acute cold stress often being essential for wild-type fitness. In general, the alteration of lipid composition in the cells and regulation of translation are the two major responses to cold stress in wide variety of taxa. The nematode *C. elegans* has been used in numerous studies on cold tolerance with various outcomes depending on the severity of the regimes. In this work, several approaches were taken to study natural variation in cold stress resistance. These included testing in novel 4-parent recombinant inbred lines (RILs), two complimentary panels of nearly isogenic lines of N2/CB4856 and wild isolates. The results uncovered extensive variation in response to cold shock treatment and, for the first time, linked cold stress survival to regulation of translation in *C. elegans*. Critically, genes previously identified in cold response cannot explain the variation detected, however, there is some evidence linking the variation to difference in *eftu-2* (a homologue of human eEF-2), a gene important for the elongation step in protein synthesis.

5. 1. Introduction

Living organisms are exposed daily to fluctuating environments. Temperature especially, is one of the most important determinant of the distribution and abundance of species (Cossins and Bowler, 1987). Unable to regulate their body temperature, poikilotherms have to adapt to cope with environmental temperature changes. The ability to match thermotolerance to seasonal stresses is an important component of fitness in an organism (Hoffman *et al.*, 2003). Temperatures below the normal temperatures for a given species, a cold stress, can impair cell function via a number of distinct mechanisms. For example, temperatures below freezing damage cells either physically via ice crystal formation (Bouvert and Ben, 2003) or biochemically via changes in solute concentration, decrease of enzymatic rates and aggregation of endogenous proteins (Kawahara, 2008). Temperatures below the thermal optima for a species, but above freezing, can also impact cell function via effects on protein function and on membrane lipid state (Guschina and Harwood, 2006).

The lipid composition of cell membranes defines a threshold level, below which membrane lipids undergo a phase transition that impairs membrane function (Hazel 1995). A higher proportion of unsaturated fatty acids lowers the temperature at which this lipid phase transition occurs, and organisms alter levels of fatty acids to promote survival at low temperatures (Cossins 1994). For example, exposure to low temperatures often increases the proportion of unsaturated fatty acids and increases subsequent ability to survive cold stress (Hazel and Williams, 1990). The nematode *Caenorhabditis elegans* displays changes in lipid saturation as an adaptation to low temperatures (Tanaka *et al*., 1996). These adaptive changes in membrane composition are mediated, at least in part, by the action of the Δ9 desaturases (Uemura *et al*., 1995; Tiku *et al.*, 1996). As expected given this situation, disrupting all three Δ9

desaturase genes in *C. elegans* reduces survival times at 4°C in both N2 and *age-1*(hx546) backgrounds (Savory *et al*., 2011), although the increased cold stress resistance that is normally seen in *age-1*(hx546) is not entirely explained by changes in Δ9 desaturase action (Savory *et al*., 2011). Little is known about the thermal range of wild type *C. elegans*, but for growth and reproduction in laboratory conditions, it is approximately 15°C-25°C (Dusenbery and Barr, 1980) with the lipid phase transition temperature occurring at approximately 10°C (Lyons *et al*., 1975). At 4°C, activity and reproduction is completely arrested, but worms can survive for prolonged periods.

The second major route by which organisms deal with cold stress is via the regulation of translation. This shows that the response to cold stress is linked to that seen in many other stresses, as the correct and appropriate regulation of translation is critical to organismal survival under many stressful conditions (Sonenberg and Hinnebusch, 2009). For example, mammalian cells exposed to relatively low temperature show progressively decreased rates of global translation (Roobol *et al.,* 2009). The reduction in global levels of translation seen in response to cell stress are generally mediated by either phosphorylation of the α subunit of eukaryotic initiation factor 2 (eIF2), or by changes in mechanistic target of rapamycin (mTOR) signalling. The translation initiation factor eIF2 functions to deliver initiator $tRNA_i^{Met}$ to the small 40S ribosomal subunit (Holcik and Sonenberg, 2005). Phosphorylated eIF2 remains bound to the exchange factor eIF2B, which prevents the eIF2-GTP-$tRNA_i^{Met}$ complex from recharging and therefore inhibits translation initiation. This results in an accumulation of stalled translation preinitiation complexes, which then aggregate to form cytosolic stress granules (SGs; Kedersha *et al*., 2002). SGs are closely related to second class of RNA granule known as processing bodies (PBs) and both are simultaneously assemlbled in cells subjected to environmental stress (Anderson and Kedersha, 2008). For example, the lowered global levels

of translation observed in human embryonic kidney cells in response to cold stress are a result of changes in the phosphorylation of eEF2 (Knight *et al*., 2015). In yeast, one of the two paralogs of eEF2 is also known to be involved in heat stress resistance (yeastgenome, 2016).

The alternative route by which a reduction in translation is produced is via inhibition of the protein kinase mTOR (Wullschleger *et al.,* 2006). The nutrient and energy status of the cell controls mTOR, acting to integrate environmental cues and regulate growth and homeostasis (Wullschleger *et al.,* 2006; Laplante and Sabatini 2012). The lack of mTOR activity leads, via the translational regulators eukaryotic translation initiation factor 4E (eIF4E) binding protein 1 (4E-BP1) and S6 kinase 1 (S6K1), to the prevention of the recruitment of 43S preinitiation complex factors and a reduction in translation (Figure 5.1; Richter and Sonenberg, 2005; Ma and Blenis, 2009). mTOR also impacts translation by promoting the phosphorylation of eEF2K leading to its inactivation (Wang *et al*., 2014). This translation suppression and the subsequent formation of SGs promote the survival of cells at low temperatures (Hofmann *et al.,* 2012). The formation of SGs and translation arrest is therefore critical to cell survival under cold stress, with compound C or Src inhibitor-1 treatment of mammalian cells under cold shock leading to a greatly increased level of cell death, an effect blocked if translation is directly inhibited by either cycloheximide or puromycin (Hofmann *et al*., 2012).

Most organisms from prokaryotes to plants and higher eukaryotes therefore respond to cold shock in a similar manner (Al-Fageeh and Smales 2006). For example, in yeast many genes involved in protein synthesis, including various ribosomal proteins, are down-regulated at temperatures below 10°C (Homma et al., 2003; Murata et al., 2006), and in *S. cerevisiae* these temperatures are also associated with prominent SGs formation (Hofmann *et al.,* 2012). In contrast, the cold adapted *S. kudriavzevii* shows an enhanced translation efficiency at low temperatures in comparison to *S. cerevisiae* (Tronchoni *et al*., 2014). Every enzymatic process

is temperature depended; therefore, subjecting an organism to cold shock forces the cellular mechanisms, including translation, to work at below optimum range. The response of *C. elegans* to cold stress is not well understood although insulin-like signalling and lipid synthesis are known to be involved (Savory et al 2011; Hu et al 2015).

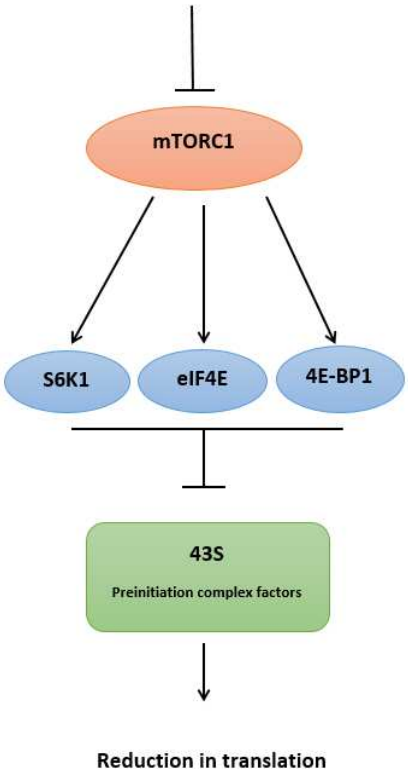


Figure 5.1 Schematic of inhibited mTORC1 activity. When mTORC1 activity is inhibited, translational regulators of eukaryotic translation S6K1, eIF4E and 4E-BP1 inhibit 43S preinitiation complex factor. This leads to a reduction in translation.

Here, I have investigated natural variation in cold stress resistance in *C. elegans* and investigated the extent to which changes in the levels of translation affect the cold shock response. Importantly, there is currently no published data about natural variation in the cold stress

response in *C. elegans*. In previous work (Chapter 5), I identified a major quantitative trait loci (QTL) that effects survival after cold shock in a new 4-parent panel of recombinant inbred lines (RILs). The analysis of genes within this QTL region rules out the genes known to be involved in the *C. elegans* cold stress response and implicates a non-synonymous substitution in *eftu-2*, the *C. elegans* homologue of eEF2.

5.2 Methods

5.2.1 Worm strains

N2, and the mutant lines: VC2595, which contains *glrx-21*(ok3427) an allele generated by the international *C. elegans* Gene Knockout Consortium (*C. elegans* Deletion Mutant Consortium, 2012), *glrx-21* (OK3427 & TM2921); VZ54, which contains *glrx-21*(tm2921) (Morgan *et al.,* 2010); RB1206, which contains *rsks-1* (ok1255); KX15, which contains *ife-2* (ok306); KX17 which contains *ife-4* (ok320) were obtained from the Caenorhabditis Genetics Centre. Wild isolates (donated by M.A. Felix to J. Kammenga, Volkers *et al*., 2013) were obtained from the Nematology Laboratory (Wageningen University, Holland). The RILs were produced and obtained from the Nematology Laboratory (Wageningen University, Holland) and obtained from J. Kammenga.

All lines were maintained on standard NGM with *Escherichia coli* OP50 as a food source (Steirnagle 2006). Assays were performed at 20°C and were initiated with fourth larval stage worms (L4s) grown from synchronized, arrested, L1s that themselves were the progeny of synchronised worms. Plates within each experiment were blind coded and treatments/genotypes randomized so that position within the incubator was not determined by treatment/genotype and that assays were scored without knowledge of worm

treatment/genotype. Plates that became contaminated with fungi were excluded.

5.2.2 Cold stress assays

All experiments were performed as described in Chapter 4, with a 4°C ±0.5°C ^{0}C cold stress of 14 days used for the analysis of wild isolates and RILs, and of 11 days for mutant lines worms. All RILs were scored within one assay, with one plate per RIL and the assay was repeated three times. There were four replicates for wild isolates scored within one assay, ten replicates for mutant lines and transgenic worms, ten replicates for N2/CB ILs and eight replicates per CB/N2 ILs.

5.2.3 QTL mapping

Mapping for response to cold stress resulted in the detection of four potential QTLs on chromosomes I and III (figure 2). These QTLs are described as csq1, csq2, csq3 and csq4, (see Chapter five for details). One of the QTLs on chromosome III (csq3) showed the largest response (LOD score 9.65).

5.3 Results

To test natural variation in cold stress response, the 4-parental RIL panel was subjected to acute cold shock of 4 °C for 14 days with a recovery period of 24 hours (This was based on Ohta *et al.*, 2014 and Robinson and Powell, 2016). The RILs show extensive variation in response to cold shock treatment (figure 5.2). Survival of the parental strains was above the average survival (38%) at 48%, 53% and 60 % (for JU1931, JU1926 and JU1941 respectively, no data for parental line JU1511 were recorded due to technical problems, namely different developmental time for this strain). N2 survival was marginally above the average for the RILs at 40%, with N2 included to allow for direct comparison with other studies. Line Z25 appeared to be an outlier

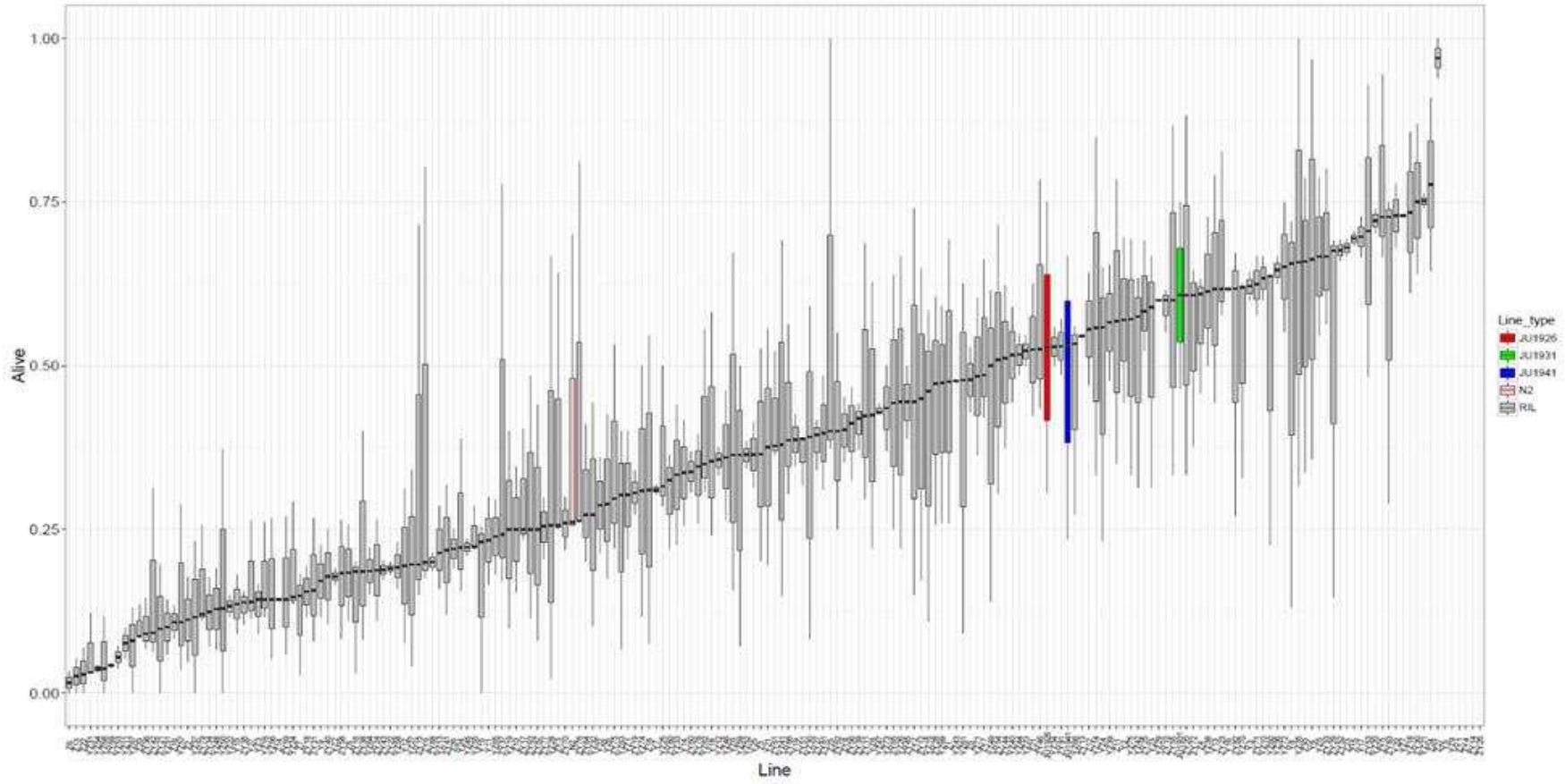


Figure 5.2 Distribution of stress response in the 4-RILs.

Shown are the ranked survival proportions of the RILs (± standard error) for cold shock treatment. The lines are sorted from the least survival to most surviving lines including parental strains JU1926 (red), JU1931 (green) and JU1941 (blue) and N2 (pink).

as nearly all of the worms (97%) survived the cold stress (for details see Chapter 4). Some of the RILs show high variability within the lines (a large standard error) which is due to the small sample sizes and to variability between assays (Figure 5.2). Bin mapping for survival of the cold shock treatment resulted in four potential QTLs, two on chromosome I (csq1 and csq2) and two on III (csq3 and csq4; Figure 5.3 & 5.4). Csq1 can be assigned to allele(s) of the JU1931 parental strain, csq2 can be assigned to allele(s) of JU1941 parental strain, csq3 can be assigned to allele(s) shared between parental strains JU1511 and JU1941 and csq4 can be assigned to allele(s) of JU1941. Detailed discussion of all cold shock QTLs can be found in Chapter 5.

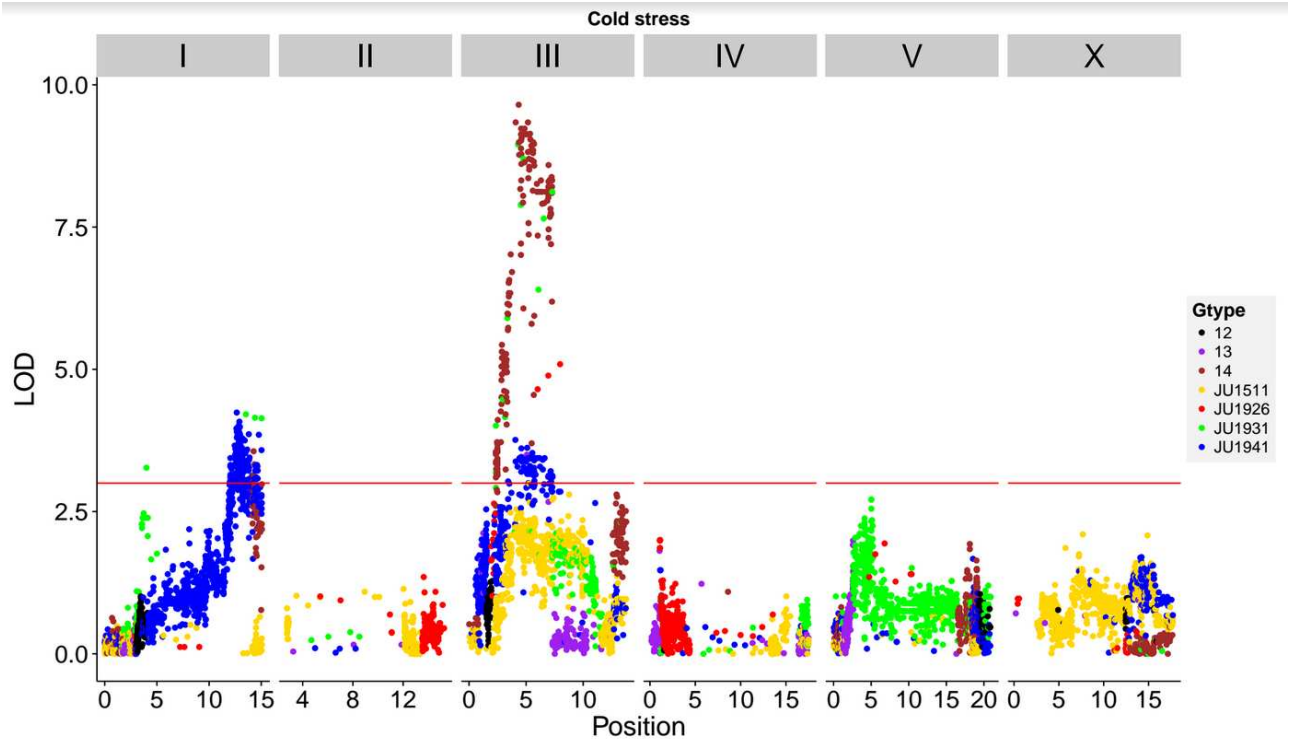


Figure 5.3 QTL mapping for response to cold shock.

The six chromosomes are shown and the red line represents a genome-wide threshold. Dots above the threshold indicates that variation at this point in the genome is associated with variation in lifespan, and indicate the presence of a QTL. These plots are coded by four parental genotypes and by combination of two parents (Gtype). Each dot represents individual SNP.

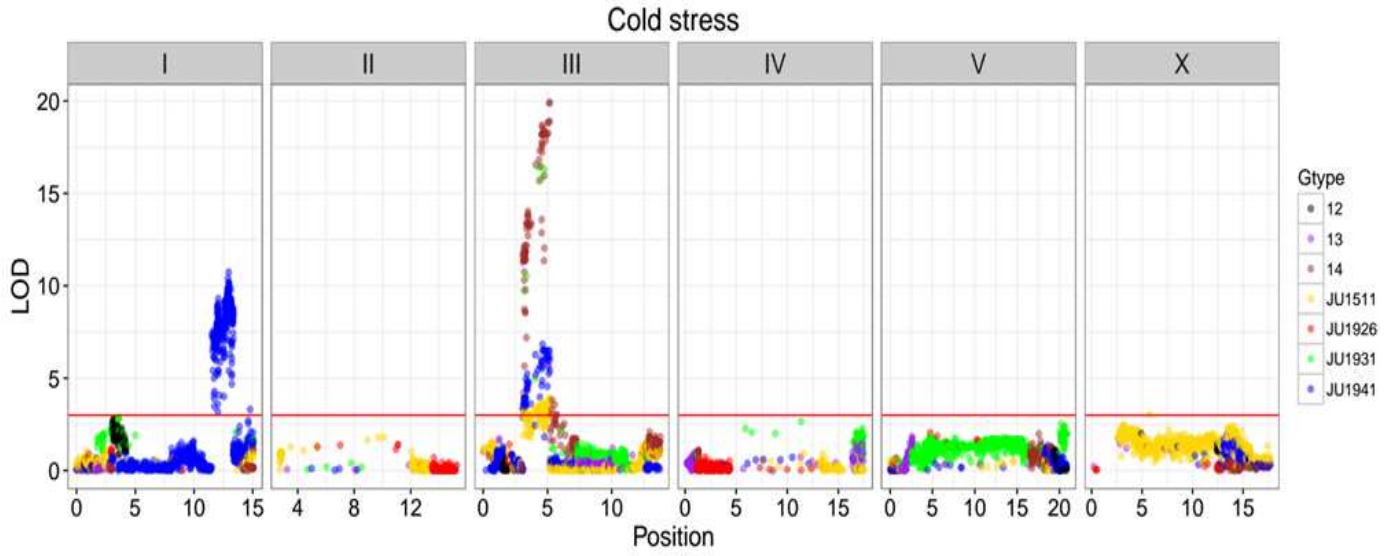


Figure 5.4 Fixed QTL mapping for response to cold shock.

The six chromosomes are shown and the red line represents a genome-wide threshold. Dots above the threshold indicates that variation at this point in the genome is associated with variation in cold stress resistance, and indicate the presence of a QTL on chromosome I (csq2). These plots are coded by four parental genotypes and by combination of two parents (Gtype). Each dot represents an individual SNP.

Because of the high LOD value of csq3, this QTL was fixed to analyse its effect on the other QTLs (Figure 5.4). This confirmed csq2 on chromosome I, but not the other two QTLs. Comparing of the alleles from the four parents reveal the difference can be assigned to allele(s) from the JU1941 parent (Figure 5.5, blue genotype).

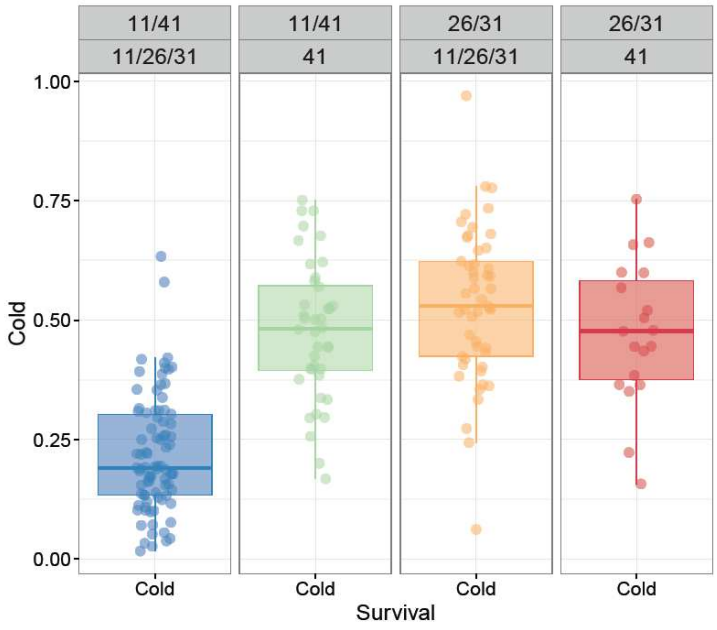


Figure 5.5 Allelic effect on survival in cold (4^{0}C for 14 days with a recovery period of 24 hours). The cold survival rates of the RILs grouped by their alleles on chromosomes I and III. The top row represents the main QTL on chromosome III and the second row represents the main QTL on chromosome I for parents (Genotypes) JU1941 (blue), JU1931 (green), JU1926 (yellow) and JU1511 (red).

To investigate to what extent cold stress survival is under balancing selection in nature, I studied this trait in 33 wild isolates collected from six different countries (Figure 5.6, Appendix E.1). Predictably, given the variation found in other traits and the variation in cold stress survival found between the parental lines of the RILs, the response in the wild isolates was very varied, from 1.5% to 95% survival. This result confirms that there is great genetic variation for survival under cold shock within wild *C. elegans* strains. All assayed strains are from a limited range of geographical locations and there is no apparent linkage of location and ability to survive cold stress. Indeed, that very different responses can be seen in strains isolated from the same region, indicates that there is no obvious local adaptation in relation to low temperature. This

outcome mirrors the conclusion of Cutter *et al.* (2006) where the results suggested that, unlike in *C. briggsae*, there was no geographical portioning of *C. elegans* in response to high temperatures.

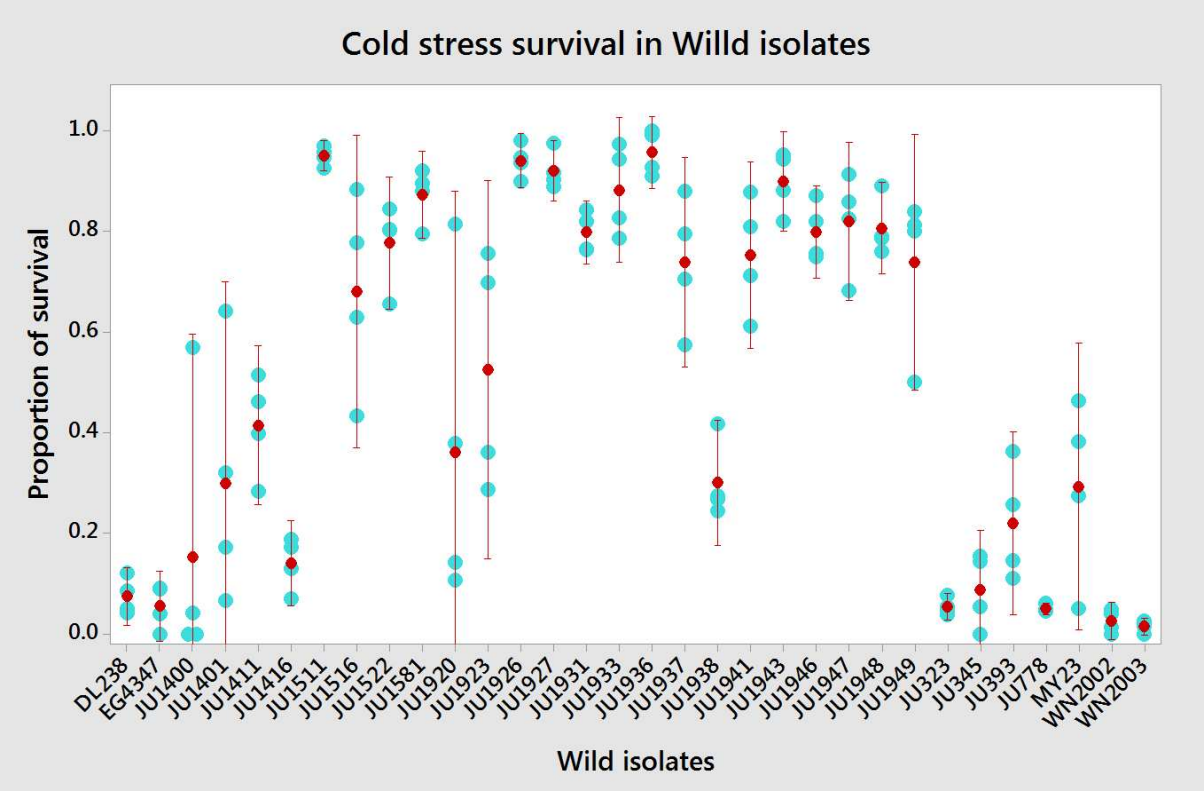


Figure 5.6 Survival under cold stress in wild isolates. Mean lifespan, ±SD is represented by red lines. Score 0 represents all worms dead and score 1 represents all worms alive.

To study if this variation of response to cold stress is present in other isolates I also determined cold stress survival in two complimentary panels of Bristol N2 and Hawaiian CB4856 nearly isogenic lines (Doroszuk *et al.,* 2009, M. Sterken, unpublished). These lines are composed of a recipient genome contributed by one of the parental strains and a short, homozygous segment of the donor genome contributed by another, genetically distinct, parental strain.

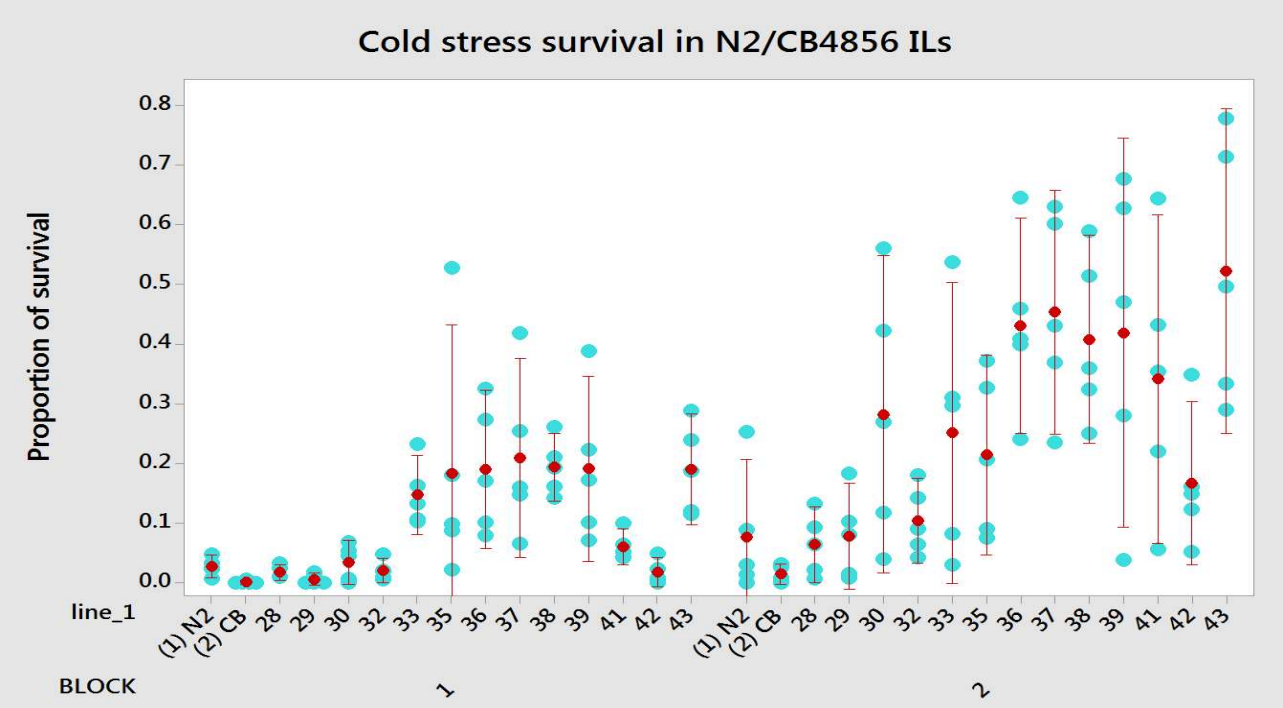


Figure 5.7 Survival under cold stress in N2/CB ILs. The cold shock survival assay was run in two separate blocks 1 and 2.

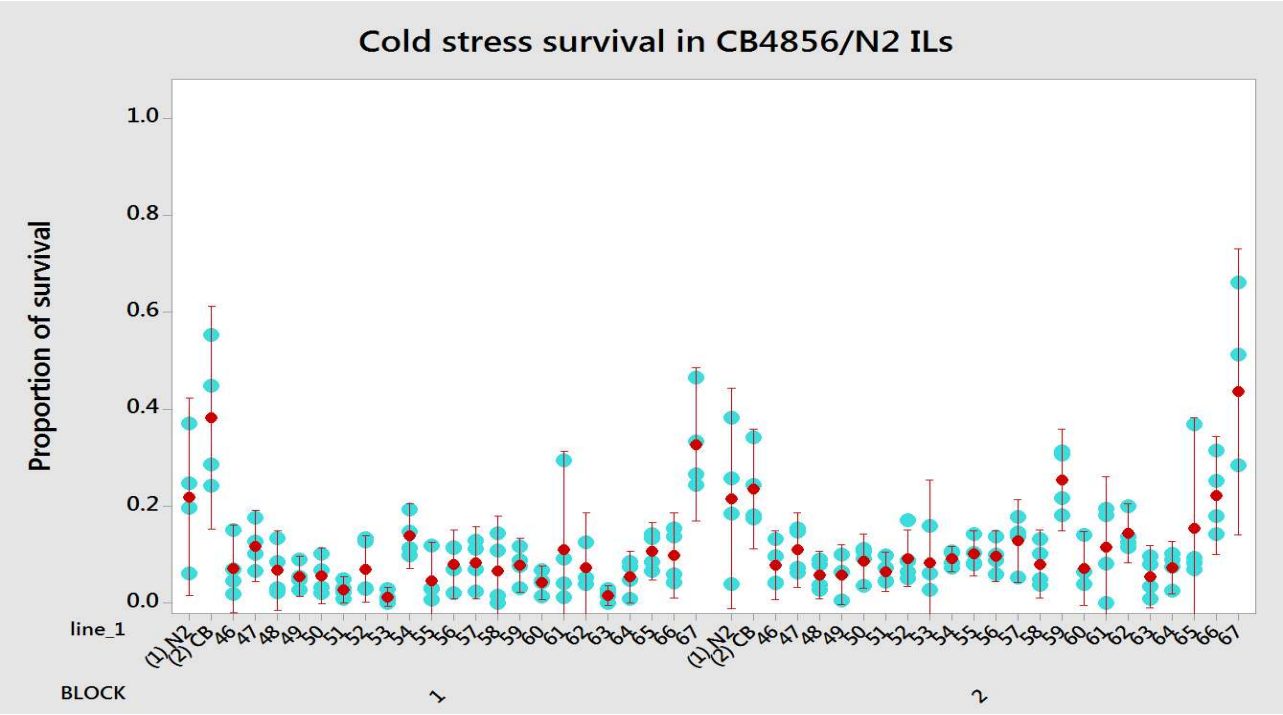


Figure 5.8 Survival under cold stress in CB/N2 ILs. The cold shock survival assay was run in two separate blocks 1 and 2.

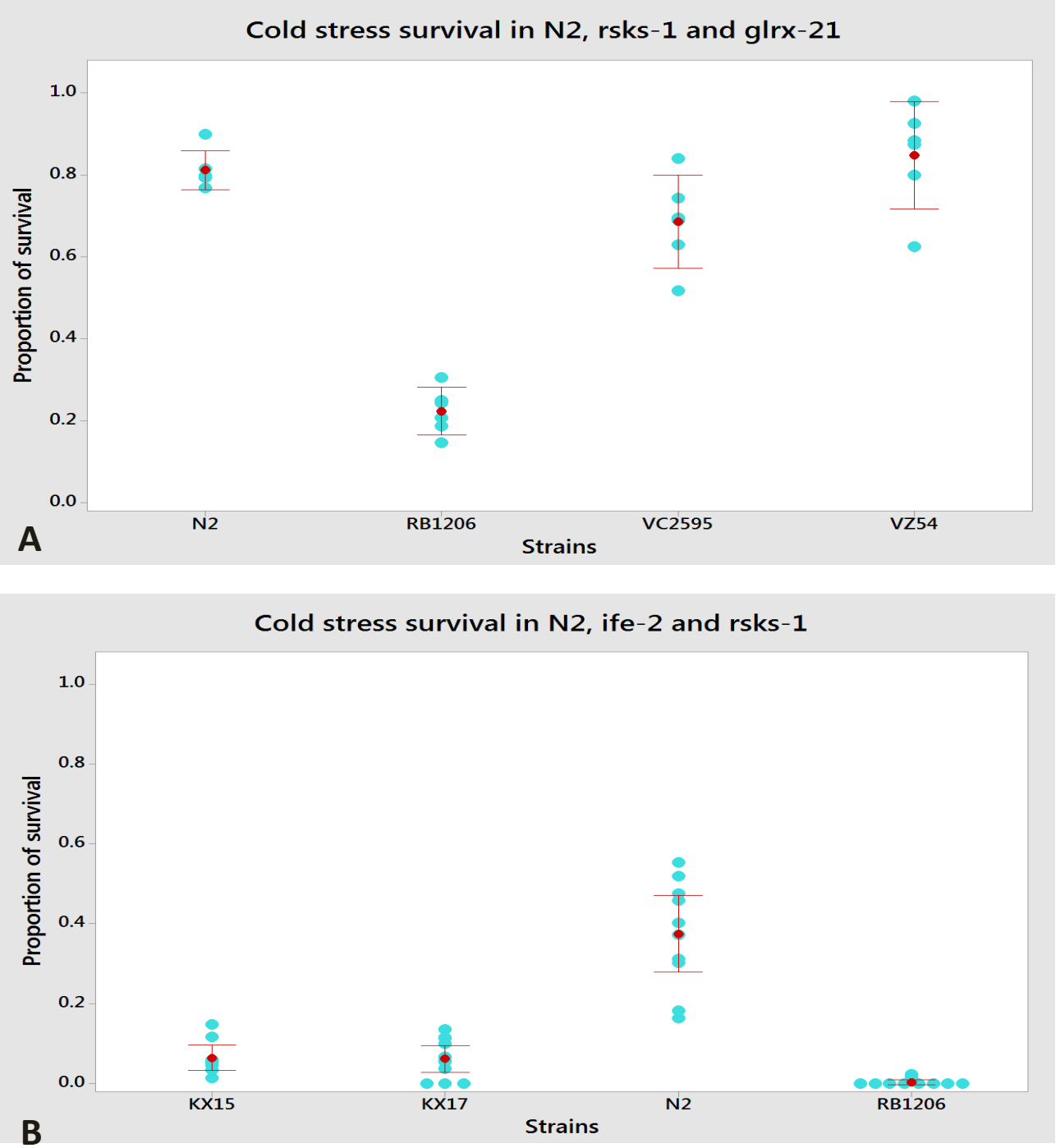


Figure 5.9 Survival under cold stress in N2 and mutants.

In box A, N2 (proportion of survival 81.1%), RB1206 (proportion of survival 22.4%) VC2595 (proportion of survival 68.6%) and VZ54 (proportion of survival 84.8%). In box B, N2 (proportion of survival 37.4%) KX15 (proportion of survival 6.4%) KX17 (proportion of survival 6.2%) and

RB1206 (proportion of survival 0.4%).

Analysis of the N2/CB ILs revealed there is a difference in response to cold stress between the two parental strains where CB (proportion of survival 0.8%) is more susceptible to cold stress than N2 (proportion of survival 5%). There was a difference between individual ILs on chromosome lll (Figure 5.6). Most of the lines were less susceptible to cold stress than the parental lines, especially, lines located around the middle of chromosome lll (lines 33, 35-39 and 43). This indicates that there are one or more CB4856 alleles within this region that increase survival of cold stress. Testing the complimentary CB4856/N2 IL panel provides general support for this – many of the ILs show a reduced survival in comparison to the parental lines, the expected result if an N2 genotype in the region reduces survival (Figure 5.7).

The next step was to look at the known genes that might affect cold stress survival. Candidates were those genes known to regulate membrane lipid composition regulation or to regulated translation. The Δ9 desaturases genes, which are known to promote survival at low temperature in *C. elegans*, were not in the QTL regions identified here. I have therefore concentrated on translation regulation, namely on available translation mutants to determine if translation is important in cold stress resistance in *C. elegans*. Viable mutations in *rsks-1*, *ife-2* and *ife-4* were selected. *rsks-1* encodes a putative ribosomal protein S6 kinase (S6K) required for normally high levels of protein synthesis, and *ife-2* and *ife-4* encode translation initiation factor 4F, cap-binding (eIF4E) subunits. To investigate further, I examined the top 200 expression QTLs (eQTLs) identified in the 4-parent RILs and located on chromosome lll for possible candidate genes. This identified *glrx-21*, a glutaredoxin oxidoreductase predicted to function in regulation of the thiol redox state of proteins, and *eftu-2*, which encodes an elongation factor 2 (EF-2) predicted to be required for the elongation step of protein synthesis

(Figure 5.10). No *daf-2* eQTL was detected and no expression differences were identified in any other candidate genes suggested in Chapter 5. No mutants of *eftu-2* are available, but a mutant allele of *glrx-21* was identified and so this was also tested (Figure 5.9). These assays indicated that the eIF4E mutants *ife-2* and *ife-4* and *rsks-1* had low survival in comparison to N2 and that survival of *glrx-21* did not differ to N2 (Figure 5.9; summarised in Table 5.1).

cold stress survival		**result**
RILs	Approx 190 lines	QTLs
N2/CB Ils	28-43	Different overall survival
CB/N2 ILs	46-67	Different overall survival
Wild isolates	Appendix E	Wide range of survival
Mutants	*ife-2*	Low survival
	ife-4	Low survival
	rsks-1	Low survival
	glrx-21	High survival
	N2	High survival

Table 5.1 Summary of cold stress assays on various lines.

This table represents a summary of all the results from various lines and strains (RILs, ILs, Wild isolates and Mutants) used for cold stress analysis. The range of survival was greatly variable ranging from zero to 100% survival.

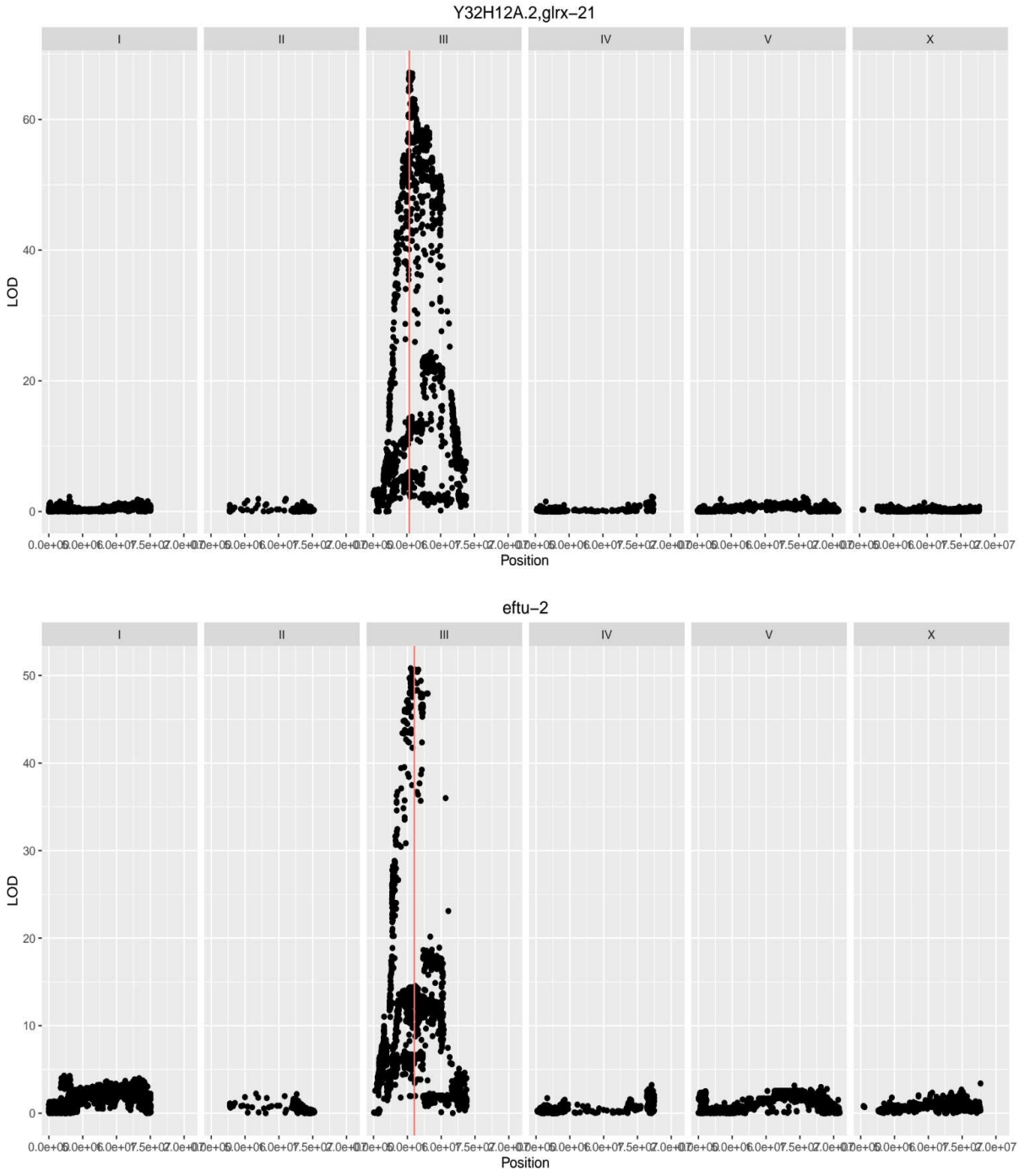


Figure 5.10 Chromosome III *glrx-21* and *eftu-2* expression Quantitative Trait Loci (eQTL).

Shown are the location of the gene (red line) and location of single nucleotide polymorphisms associated with expression differences in the gene. In both cases, this analysis indicates local (cis-acting) regulatory control.

The next step was to search WormQTL^{HD} (Human Disease) database for any further evidence for *eftu-2*. WormQTL^{HD} is a database that quantitatively and systematically links expression Quantitative Trait Loci (eQTL) findings in *C. elegans* to gene–disease associations in humans is available online at http://www.wormqtl-hd.org (van der Velde *et al.*, 2014). Search in WormQTL^{HD} revealed an eQTL in *eftu-2* in a number of assays of N2/CB4856 RILs (Figure 5.11).

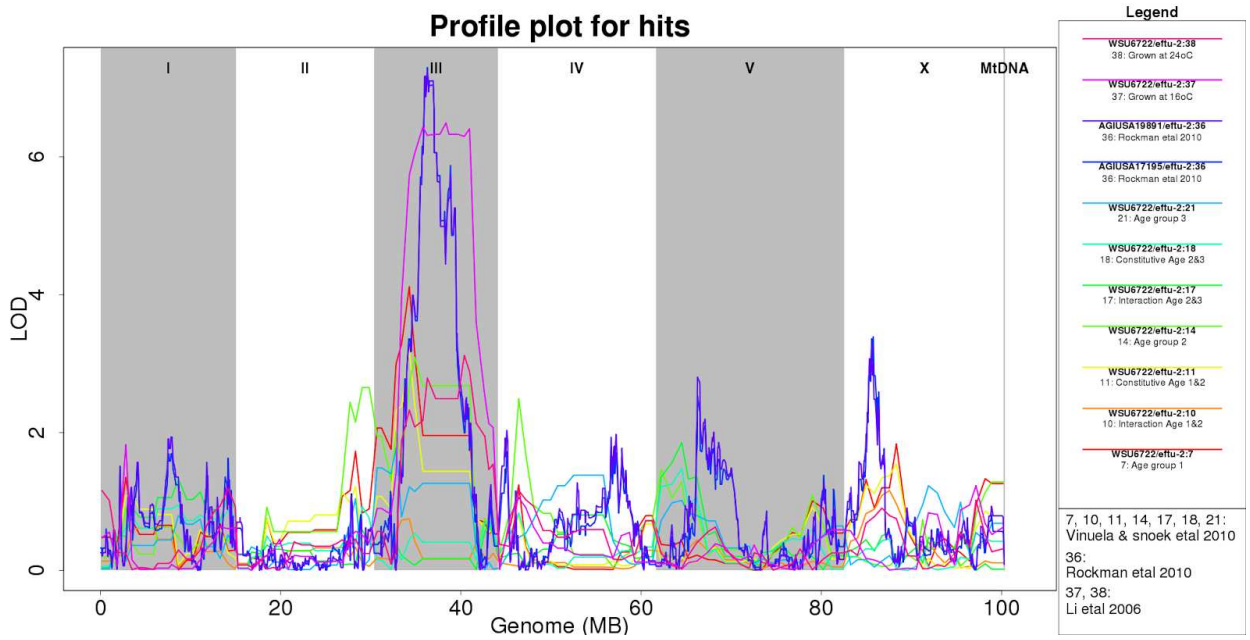


Figure 5.11 Detection of *eftu-2* expression in various studies from WormQTL^{HD}.

The colour-coded lines represent *eftu-2* eQTL expression from different experiments. Of iimportance are orange and red colour representing difference in gene expression at two distinct temperatures (24°C and 16$^{°C}$ respectively).

6.4 Discussion

Here I have shown, in wild isolates, a wide spectrum of responses to acute cold stress. The analyses of *C. elegans* from the 4-parental RILs panel subjected to acute cold shock of 4$^{°}$C for 14 days display highly varied response in survival. The analyses uncovered four major cold shock

QTLs on chromosomes I and III. Further analysis of these data supported two of these QTLs. Two complimentary panels of N2/CB4856 nearly isogenic lines were also tested for cold shock survival, again uncovering differences in survival related to variation on chromosome III. These analyses indicate that at least one gene on chromosome III is a major regulator of survival in response to cold stress in *C. elegans*. One of the interesting outcomes of this analyses is there was no detection of genes previously identified to play an important role in response to cold treatment such as *ucp-4* (Iser *et al*., 2005) or of *fat-5, -6 or -7*(Savory *et al*., 2011). All of these genes are located on chromosome V, however, the cold shock QTLs were detected on I and III. Consideration of other candidate genes on chromosome III presents a limited number of plausible genes, with the eQTL results suggesting that the variation in cold stress survival might be a consequence of variation in *eftu-2* (a homologue of human eEF2).

Given the widespread role of translation-regulation in the response to cold stress a number of lines with loss-of-function mutations in components of the translation machinery were therefore tested. This indicated that deletion of the S6K *rsks-1*, and of the eIF4E/*ife-2* genes *ife-2* and *ife-4*, which encode mRNA cap-binding protein subunits, all lowered survival under cold stress when compared to that of loss-of-function mutants in the oxidoreductase *glrx-21* and to N2 (Table 5.1). This indicates that translation regulation is critical to cold stress survival in *C. elegans.*

In *C. elegans*, habituation to cold temperature seems to be the key to survival as exposure to low, but non-stressful, temperatures can improve cold tolerance. For example, worms cultivated at 15°C can survive at 2°C and 4°C, whilst those grown at 20°C can survive at 4°C (Ohta *et al*., 2014). Also, worms cultivated at 15 °C have increased longevity, which is at least partly regulated by the co-chaperone p23 (Horikawa *et al.,* 2015). In this study, deletion of co-chaperone p23 led to increased survival at high temperatures whether at low temperatures the

worms were short lived. However, the survival itself although important must be followed by process of recovery that occurs for up to several days after the cold shock (Clark and Worland, 2008; Robinson and Powell, 2016). Understanding of cold stress recovery in *C. elegans* is still very poor. For example, in *D. melanogaster* and *S. cerevisiae* whilst there are some genes directly involved in cold shock survival, most changes in gene expression occur after the return to normal temperatures (Collinet *et al.,* 2010; Al-Fageeh and Smales 2006). These studies seem to confirm that cold stress recovery is genetically controlled.

Importantly, as nematodes are found on all continents and are greatly diverged in their thermal niches, it should also be possible to identify adaptations that would allow their cellular machinery to function at widely different temperatures. There is therefore the potential to extend the above analyses to a wider range of polymorphisms. Understanding of cooling of mammalian cells is crucially important for a variety of medical reasons and has potential importance in a number of fields. For example, in improving cryopreservation of cells important for stem cell storage or assisted reproduction (Pegg, 2007), for operations requiring deep hypothermic circulatory arrest (DHCA; Conolly *et al*., 2010), used in transplant medicine (Berendsen *et al*., 2014) and industrial application in recombinant protein production (Al-Fageeh *et al*., 2006).

CHAPTER SIX: Analysis of various dietary restriction regiments

Abstract

Dietary restriction (DR) is considered a near universal means of lifespan extension in animals. In addition to extending lifespan, DR has been shown to prevent age-related diseases such as cardiovascular disease, diabetes and dementia. DR therefore appears to act generally in keeping organisms relatively healthy until older age. In *C. elegans*, DR has been extensively investigated in a single genetic background (N2) using a wide range of methods. Although this work has been important in developing our understanding of the mechanisms by which DR extends lifespan, it does not tell us about how these responses may differ in different genetic backgrounds. Here, I present an analysis of lifespan under normal and multiple DR conditions in selected lines from multi-parent recombinant inbred lines (RILs) derived from wild isolates. My analyses show there is extensive variation in the response to DR and that this varies by method of DR and by day of administration .

6.1 Introduction

Reduction of food intake, whilst avoiding malnutrition, can ameliorate ageing and ageing-associated diseases in invertebrate model organisms as well as rodents, monkeys and humans (Fontana and Partridge, 2015). There seems to be overwhelming evidence that the life extension results from slowing of the ageing process due to dietary restriction (DR, Fontana *et al*., 2010). Animals subjected to DR live longer, appear to be more youthful and are protected from the common diseases of ageing (Weindruch and Walfrord, 1988). Yet, the mechanisms underlying dietary restriction are still not fully understood and an important question, of how the system works, remains. It is also clear that DR can act in a genotype-specific manner and that it does not always extend lifespan (see Chapters 2 and 3 for *C. elegans* data supporting this and Chapter 1 for a review of other literature). The fact that many of the longevity-extending mutations appear to lower the activity of nutrient-signalling pathways suggests, that they promote a specific physiological state that is similar with the fast and feast of natural cycles that animals can experience in nature.

A variety of DR regimens have been described in a species ranging from yeast to humans (Stastna *et al*., 2015; Schleit *et al*., 2013; Mair *et al.,* 2005; Rusli *et al*., 2015; Mattison *et al*., 2012; Colman *et a*l., 2009; Cava and Fontana 2013; for review see Fontana *et al*., 2010). Multiple methods of DR have been used in *C. elegans* which allow for some comparison between the various regimens (Table 6.1). These analyses have identified a number of evolutionary conserved pathways including; mechanistic target of Rapamycin (mTOR), AMP-activated protein kinase (AMPK), Sirtuins, and insulin/insulin-like growth factor (IGF-1) signalling (Olshansky *et al*., 2016). Interestingly, work on DR has shown, both within *C. elegans*

Metdod	Genetical intervention	Bacterial dilution	Bacterial dilution	Bacterial dilution	Bacterial deprivation	Peptoe dilution	Total starvation	Intermitted fasting
Medium	agar plates	liquid culture	liquid culture	agar plates	agar plates	agar plates	agar plates	agar plates
Food source	eat-2 mutant	heat killed *E. coli*	live *E. coli*	live *E. coli*	UV killed *E. coli*	live *E. coli*	No *E. coli*	live *E. coli*
% of LS extension	57	128	28	18-35	51-67	23.5	13.5-42.5	40.4-56.6
Use of FUdR	FUdR	FUdR	FUdR	No FUdR	FUdR	No FUdR	FUdR	FUdR
Day initiated	day 1 of adulthood	L4 stage	L4/young adult	Day 4 of adulthood	L4 stage	L4/young adult	L4/young adult	L4/young adult
Reference	(a)	(b)	(c)	(d)	(e)	(f)	(g)	(h)

Table 6.1 Different methods of DR in *C. elegans*. References: (a) Hansen *et al*. (2006); (b) Houthoofd *et al*. (2003); (c) Bishop and Guarente, (2007); (d) Greer *et al*. (2007); (e) Smith *et al*. (2008); (f) Hosono *et al.* (1989); (g) Lee *at al*. (2006); (h) Honjoh *et al*. (2009).

and more generally, that various DR methods operate via independent and/or partially overlapping pathways (Greer and Brunet, 2009).

A good example of this is shown by comparing the effects of different DR mechanisms on *C. elegans*. Here, one commonly used method of DR is the use of mutant worms that are unable to feed effectively (*e.g. eat-2* loss-of-function mutants). These *eat* mutants have reduced pharyngeal pumping that reduces food intake, leading to worms that generally present a starved appearance (Avery 1993, Hansen *et al.,* 2007), and that show a lifespan extension that depends on the severity of mutation (Lakowski and Heikimi, 1998). The lifespan extension seen in *eat* mutants however appears to be independent of DAF-16 function and possibly distinct from insulin/IGF-1 signaling (Walker *et al*., 2005). To further illustrate the complexity of the control of the response to DR, it appears that strong-effect *eat* mutants are regulated by mTOR (Hansen *et al*., 2007), whilst weak-effect *eat* mutants may be regulated by the sirtuins (Rogina and Helfand, 2004).

More generally in *C. elegans*, DR is often achieved by reducing food availability, with this achieved via a range of routes and severities: from mild DR, such as peptone withdrawal (Hosono *et al.,* 1989; Stastna *et al.,* 2015), to severe regimens such as total starvation (Lee *et al.,* 2006; Greer *et al.,* 2006; Smith *et al.,* 2008;). Reducing or total withdrawal of peptone on the plates results in limited bacterial growth. This method is fairly mild form of DR (Chapter 2/Stastna *et al*., 2015, Table 6.1). Reducing live bacteria on the plates can be also achieved by serial dilution and resuspension of bacteria in S-medium to inhibit bacterial growth, this method seems to operate via the AMPK pathway (Greer *et al*., 2007). Feeding worms with UV-killed bacteria is another widely used method with results suggesting that the lifespan extension is partially calorie-independent (Smith *et al*., 2008). Total food withdrawal, also

called bacterial deprivation, is another recently used method of DR (Kaeberlein *et al*., 2006; Lee *et al*., 2006). This method seems to increase average lifespan up to 50% (Kaeberlein *et al*., 2006), which is a clear demonstration that long-term starvation can result in lifespan extension in *C. elegans*. Bacterial deprivation, although related to *daf-2* and *daf-16* signaling, extends lifespan independently of insulin signaling (Kaeberlein *et al*., 2006; Lee *et al*., 2006). Intermittent fasting is very effective method in many divergent species and has been, until recently, notably absent from *C. elegans* studies. This regimen of an alternate-day fasting or every 2 days fasting has been shown to increase lifespan significantly as well as increasing resistance to heat and oxidative stress (Honjoh *et al*., 2009; Table 6.1). Molecular mechanisms of this regimen are still largely unknown, however RHEB-1 seems to have a dual role in lifespan regulation (Honjoh *et al*., 2009).

Another method of DR is reducing food availability in liquid-based conditions. There are several approaches such as using bacterial dilution in S-basal medium or axenic culture (Klass, 1977; Houthoofd *et al*., 2003; Bishop and Guarente, 2007). These methods are not commonly used for ageing studies, possibly due to the fact that a few early studies reported delayed development and slow growth in such conditions (Johnson *et al.,* 1984; Vanfleteren and Braeckman, 1999) or because observing lifespan of worms in the liquid culture is more difficult than on the petri plate.

All of the above methods, useful as they are, have used the canonical strain N2 and/or its derived mutants. One of the main characteristics of N2 is its 'placid' nature. By this, I mean that N2 does not aggregate on food (Weber *et al*., 2010; Sterken *et al*., 2015), making it easy to manipulate and pick the individual worms. In addition, this 'wild type' does not try to escape from the petri plates unlike the freshly-collected wild isolates or burrow into the agar. In

contrast, wild-derived isolates are difficult to culture in the laboratory (presenting a possible reason as to why they have been mostly ignored in DR studies) due to aggregation behavior, increased burrowing and increased escaping from the petri plates, all traits that are increased under conditions of DR.

For these reasons, methods of exploring various types of DR in the 4-parent RILs derived from *C. elegans* wild isolates were rather limited. In this Chapter I explore various DR regimens available for *C. elegans*. I explore the use of total starvation in liquid culture initiated at day 1 of adulthood. Additionally, I use total peptone withdrawal as described previously in Chapter 2/Stastna *et al*. (2015) as a treatment, as well as way to administer more severe levels of bacterial dilutions. To uncover to what extend does DR treatment prolongs lifespan depending on initiation date, I will test these regimes at day 1 and day 4 of adulthood.

6.2 Methods

6.2.1 Worms

To test the various DR regimens, I selected thirteen lines based on previous lifespan and DR results (as described in Chapter 4). These lines were the six lines showing the largest positive response to DR, ZY62 (+6.96 days), ZY33 (+6.93 days), YZ69 (+6.9 days), YZ39 (+6.68 days), Z1 (+6.1 days) and ZY44 (+6.07 days) and the six lines showing the greatest reduction in lifespan in response to DR, Y21 (-6.3 days), YZ18 (-5.63 days), ZY76 (-5.4 days), Z11 (-5.0 days), ZY17 (-4.07 days) and YZ67 (-4.0 days), and the very long-lived Y16 (average normal lifespan 26.27, lifespan under DR 27.29 days). N2 was also included as a control. I have used all above lines for experiment one, total starvation. For experiment two I selected five lines Y21, YZ67, Z1, Z11

and ZY76 and N2 to use in multi-method DR experiments. All lines were maintained at 20°C on standard nematode growth medium (NGM) with *Escherichia coli* OP50 as a food source (Stiernagle, 2006).

6.2.2 Total Starvation Assay

Synchronized populations of *C. elegans* were cultured at 20°C in nematode growth medium as described previously (Stiernagle, 2006). On the first day of adulthood, animals were washed three times with sterile water, then washed three times with M9 buffer, and then centrifuged at 2500mp for two minutes. After the last spin, the worm pellet was re-suspended in S-Complete and with ampicillin at a concentration of 10 µl/ml final concentration. Worms were then transferred into 96 well plates and 10 µl of 0.6 mM FUdR was added in each individual well. An average of 20 animals were used per well with a final volume of 50ul. Worms were scored daily for well activity read-out using WMicrotracker-One™ (Phylum Tech, Sunchales, Argentina) with 8 wells per line and at least three replicates. Motility assessments were conducted in an incubator at 20 °C.

6.2.3 DR assays on plates

Worms were grown *en masse* to adulthood and eggs were collected from sodium hypochlorite treated gravid adults (Stiernagle, 2006). These eggs were then maintained for 24 hours without food at 20°C. After this, the synchronized L1s were fed the OP50 *E. coli* and left to develop to L4/early-adulthood. At this point, worms whose treatment was initiated at day one of adulthood were transferred to fresh 35mm plates (n = 100 per treatment per genotype, with 10 worms per plate for all three DR treatments (dauer plates, bacterial dilution and starvation). Control, *ad libitum* food, worms were maintained on standard NGM plates. Worms for DR initiation at day 4 were cultured on NGM plates with OP50 *E. coli* until day 4 of adulthood as

described in Greer *et al*. (2007) and then transferred to fresh 35mm plates (n = 100 per treatment per genotype, with 10 worms per plate for all DR treatments). All worms were moved every other day to fresh plates for the rest of their lives to eliminate confounding progeny and, for the various DR treatments, to maintain food availability. Worms were scored daily as dead if they did not respond to a gentle touch with a platinum pick, worms that crawled of the plates or died of bagging phenotype were censored out of the experiment. No FUdR was used in these experiments. All DR treatments on petri plates were started from the same batch of worms and were conducted at the same time.

Mild DR treatment (dauer plates) was achieved by total peptone withdrawal from standard NGM plates (Hosono et al., 1989; Stastna et al., 2015), a condition that stops bacterial growth on the plates, yet does not kill the bacteria. Plates were prepared as a standard NGM only without addition of peptone. Two day old plates were than seeded with OP50 *E. coli* and left for a further two days at room temperature. This step is necessary in order to exclude any plates where OP50 *E. coli* was actively growing from the experiment. For more severe DR treatments, two different bacterial dilutions, $5x10^9$ and $5x10^8$ bacteria/ml, were prepared on total peptone withdrawal NGM plates as described above, with 2-days old plates inoculated with the bacteria and plates incubated for 2 days at room temperature. Bacterial dilutions were prepared from cultures incubated at 37 ^{0}C for 14 hours at 250rpm, with bacteria centrifuged to pellets, the supernatant discarded and the pellet re-suspended in S-medium. This was repeated three times before serial dilutions were performed to achieve the requisite cell density.

6.2.4 Data analysis.

Statistical analysis was carried out by Kaplan-Meier survival plot with Log-Rank test. All of these analyses were done in Minitab® Statistical Software (Mintab Ltd., Coventry). Lifespan in the RILs was analyzed using Survival curves in R Project for Statistical Computing (version 3.1.3, R

Foundation for Statistical Computing, Vienna, Austria, (URL http://www.R-project.org/). Survival of the worms in the starvation assay in liquid culture was analyzed using read-outs from the WMicrotracker-One™ (PhylumTech), which records movement as photo-beam interruptions (bins) within wells of 96-well plates. These data were than compiled and analysed with one-way ANOVA, the survival was calculated as 50% survival rate.

6.3 Results

6.3.1 Total starvation in liquid culture

DR in *C. elegans* can be applied by many different methods. Here, in the first experiment, I tested response of selected 4-parent RILs to total starvation in liquid culture. Response was assessed as the time to a 50% reduction in motility as assessed by the WMicrotracker-One™. This indicated that, on average, a higher activity of worms in the wells was associated with a longer lifespan in previous assays (Table 6.2, Figure 6.1).

When activity rate under total starvation was compared to average lifespan (extracted from Chapter 4), all of the lines except YZ18 appeared to have higher survival under total starvation. To allow for this comparison, I tested whether the mean lifespan correlates with 50% survival (see Appendix F, Figure F1 and Table F2). I measured the lifespan to first 50% death in N2, the resulting number was then compared with the measurements for mean lifespan. The results suggest that this comparison is indeed possible with ±1-day error.

It appears that the lines in which previous DR treatment extended lifespan had significantly higher activity under the total starvation regime (22.45% - 121.6%) when compared to lines in which DR treatment shorten lifespan (-2.23% - 38.03%; Table 6.2). N2 displayed 35% increase

Line	Activity	50% activity	Lifespan	% difference	LS under DR
YZ39	34.82	16.8	13.72	22.45	extended
YZ18	34.56	18.4	18.63	-2.23	shortened
YZ69	34.38	19.8	12.72	55.66	extended
YZ67	42.68	24.5	21	16.37	shortened
ZY44	37.92	25.3	14.57	73.64	extended
N2	48.95	27	20	35	Control
ZY76	46.87	27.6	20	38	shortened
ZY17	35.75	27.8	22.36	24.33	shortened
Z11	46.55	28	20.625	35.76	shortened
Z1	47.23	28.1	14.5	93.79	extended
Y16	41.04	28.5	26.27	8.49	extended
Y21	39.3	29.4	21.3	38.03	shortened
ZY33	48.97	30	13.538	121.6	extended
ZY62	50.21	30.3	15.12	100.4	extended

Table 6.2 Results of total starvation. Recorded was mean average activity in the well; time, in days, to 50% activity; Lifespan is the mean lifespan in days for *ad libitum* fed worms from Chapter 4; LS under DR represents the effect of DR on the line, again taken from Chapter 4 and indicates whether DR extended or shortened lifespan.

survival rate, however, the extremely long-lived line Y16 displayed only 8.49% increase. This outcome is suggestive of an earlier idea that if an individual is extremely long-lived, DR alone cannot further prolong the mean lifespan. The reason for thinking like this is, there is a possibility that the lifespan extending biochemical pathways modulated by DR are already maximally modulated in strains that are naturally long-lived (Liao et al., 2010).

Based on results from total starvation experiment, lines Y21, YZ67, Z11 and ZY76 that were originally shorter lived under mild DR treatment of peptone withdrawal on NGM plates were selected for second set of experiments on severity of DR treatments. Also included was line Z1 as an example of lifespan extension under DR treatment and to allow for comparison between treatments and N2 for control.

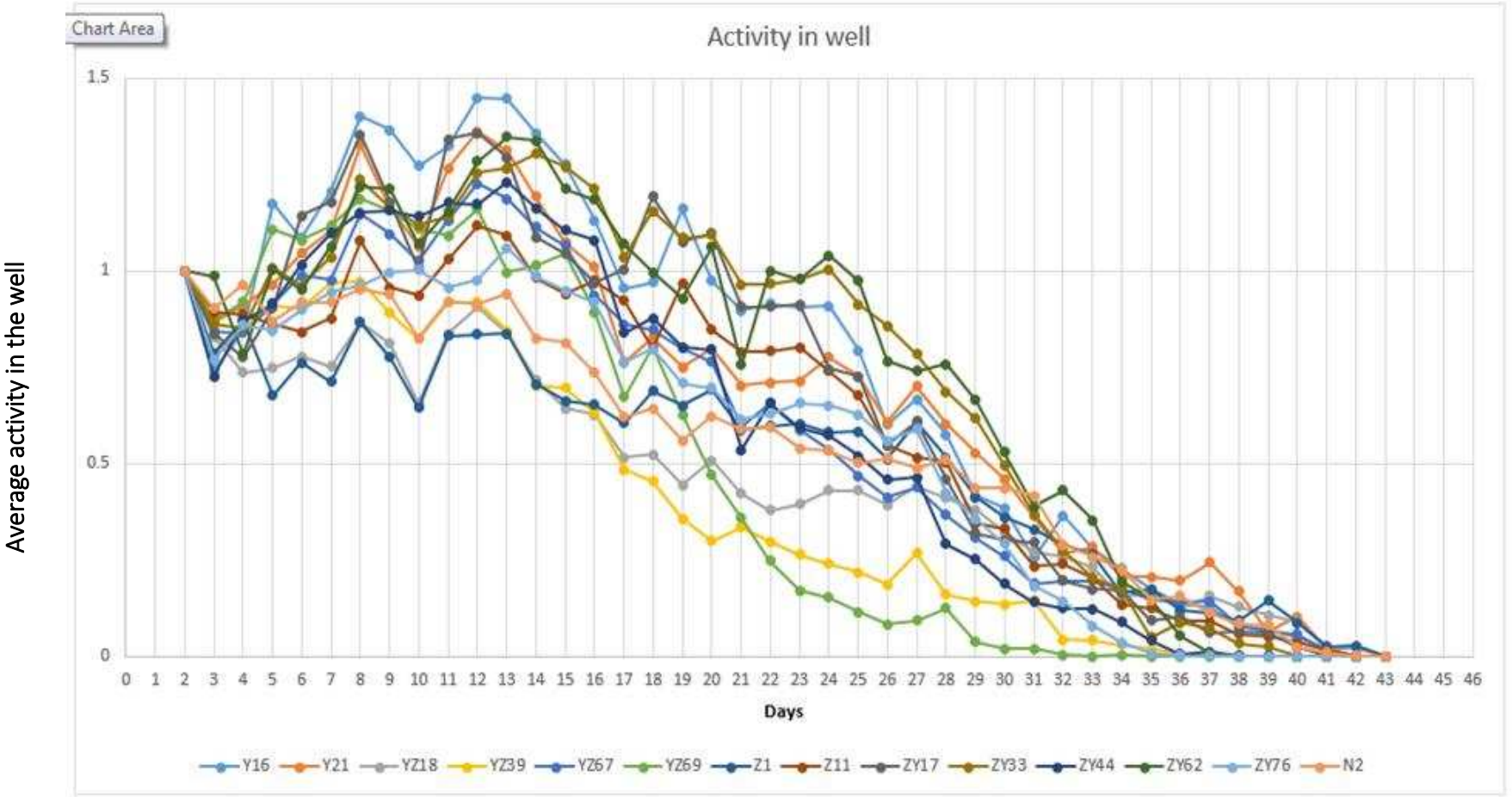


Figure 6.1 Average activity of worms in the 96 well plate.

Each line represents average activity for individual RIL plus N2. 1 represents average activity on day 2 of the experiment under conditions of total starvation.

6.3.2. Various DR treatments

Analysis of the response of selected 4-parent RILs to various severities of DR applied at day 1 or day four of adulthood revealed that, overall, all treatments extended mean lifespan (3.5 - 82.3% Table 6.3). In general, the increase of lifespan was greater when initiated at day one of adulthood (37.5 – 82.3 %) when compared to day four initiation (3.47 – 50.3 %).

	Lifespan (days) day 1 initiation				Lifespan (days) day 4 initiation		
Line	NGM	Peptone withdr.	Bac. Dil.	Starv.	Peptone withdr.	Bac. Dil.	Starv.
Y21	18.0±4.6	28.9±4.2	29.3±6.5	29.3±5.7	24.1±6.4	22.9±5.5	27.0±10.7
% LS increase	CONTROL	61.02*	64.25*	64.25*	33.24*	27.62*	50.33*
YZ67	18.9±3.5	27.5±4.9	28.9±5.9	33.5±5.5	25.1±6.1	23.1±4.1	23.3±5.6
% LS increase	CONTROL	49.32*	57.26*	82.33*	36.54*	25.39*	26.59*
Z1	19.9±6.0	28.1±4.8	29.4±5.8	27.3±6.5	24.4±6.3	23.4±6.8	27.1±7.6
% LS increase	CONTROL	41.25*	47.79*	37.53*	22.94*	17.61*	36.32*
Z11	18.5±3.0	27.0±7.1	31.9±8.9	30.3±10.2	19.1±6.4	26.5±6.4	20.2±7.4
% LS increase	CONTROL	46.34*	72.68*	64.07*	3.47	43.47*	9.49
ZY76	19.9±4.4	27.6±5.7	31.9±4.4	29.7±7.5	23.5±4.2	24.5±4.3	23.8±5.6
% LS increase	CONTROL	38.15*	59.85*	49.02*	17.84*	23.01*	19.45*
N2	16.8±3.8	24.1±2.8	27.2±3.1	24.5±4.3	21.0±3.8	22.2±3.7	20.4±3.5
% LS increase	CONTROL	43.25*	61.93*	45.57*	24.93*	32.3*	21.18*

Table 6.3 Lifespan and the effect of various severity of bacterial dilution. Displayed is mean lifespan ± St Dev and percentage of lifespan increase under various DR treatments. * denotes cases where the treatment compared to control (feeding *ad libitum*) was significantly different.

I have previously reported life-shortening effect of DR treatment in about third of 4-parent RIL panel. Several of these lines were selected and re-tested for varied severity of DR. Line Y21 was

originally shorter-lived under mild DR (-29.6%; peptone withdrawal; Chapter 4). I have not observed shortening of lifespan in this line under any of the DR conditions (Table 6.3, Figure 6.2). Here, significant life extension was recorded for, peptone withdrawal (61% increase, Log-Rank p-value <0.001), bacterial dilution (64% increase, p-value <0.001) and starvation (64% increase, p-value <0.001) treatments initiated at day one of the adulthood. Initiation of DR at day 4 of adulthood also resulted in extending of lifespan for peptone withdrawal (33% increase, p-value <0.001), bacterial dilution (28% increase, p-value <0.001) and starvation treatment (50% increase, p-value 0.006). 50% survival was calculated from survival plot to allow comparison with total starvation assay.

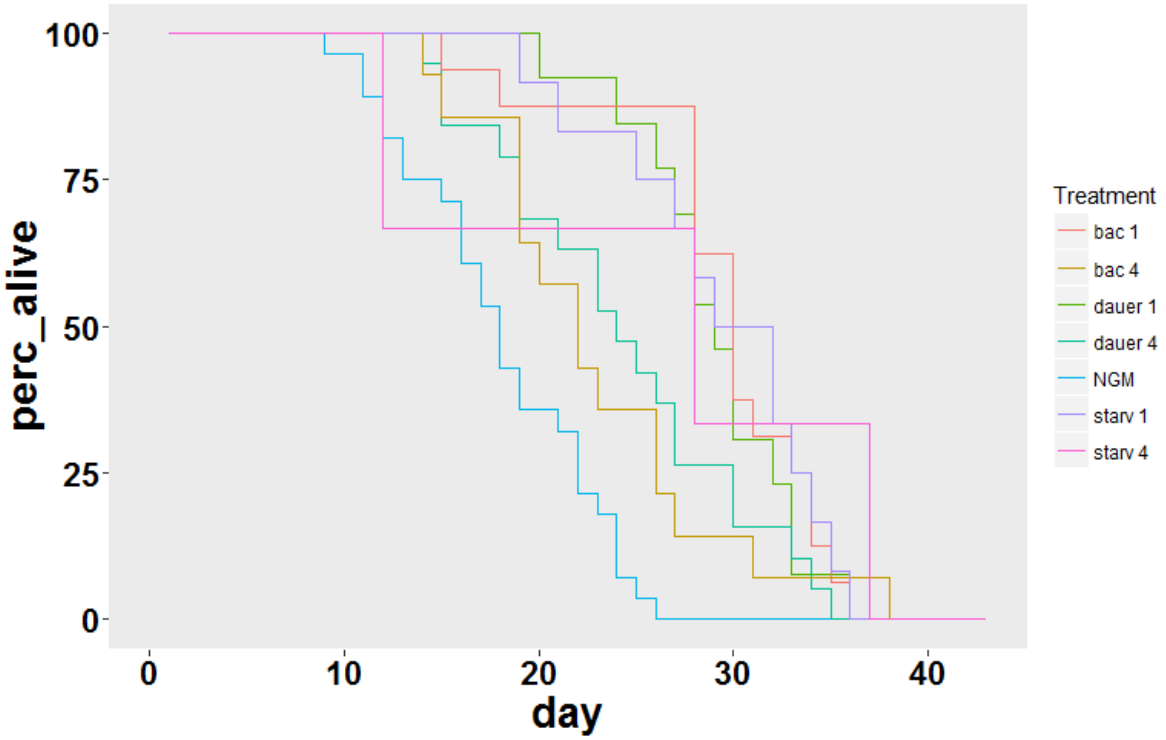


Figure 6.2 Various DR treatments for Line Y21. Normal lifespan (NGM), peptone withdrawal (dauer), bacterial dilution $5x10^9$ (bac) and bacterial dilution $5x10^8$ (starv) on day 1 and day 4 of adulthood.

Line YZ67 was originally shorter-lived under mild DR (-19.1%; peptone withdrawal; Chapter 4). Again, I have not observed shortening of lifespan in line 91 under any of the DR conditions (Table 6.3, Figure 6.3). Here, significant life extension was recorded for peptone withdrawal (49%, Log-Rank p-value <0.001), bacterial dilution (57%, p-value <0.001) and starvation (82%, p-value <0.005, SD 6.05) treatments initiated at day one of the adulthood. Initiation of DR at day 4 of adulthood also resulted in extending of lifespan for dauer (37%, p-value <0.001), bacterial dilution (25%, p-value <0.001) and starvation (27%, p-value <0.001) treatments.

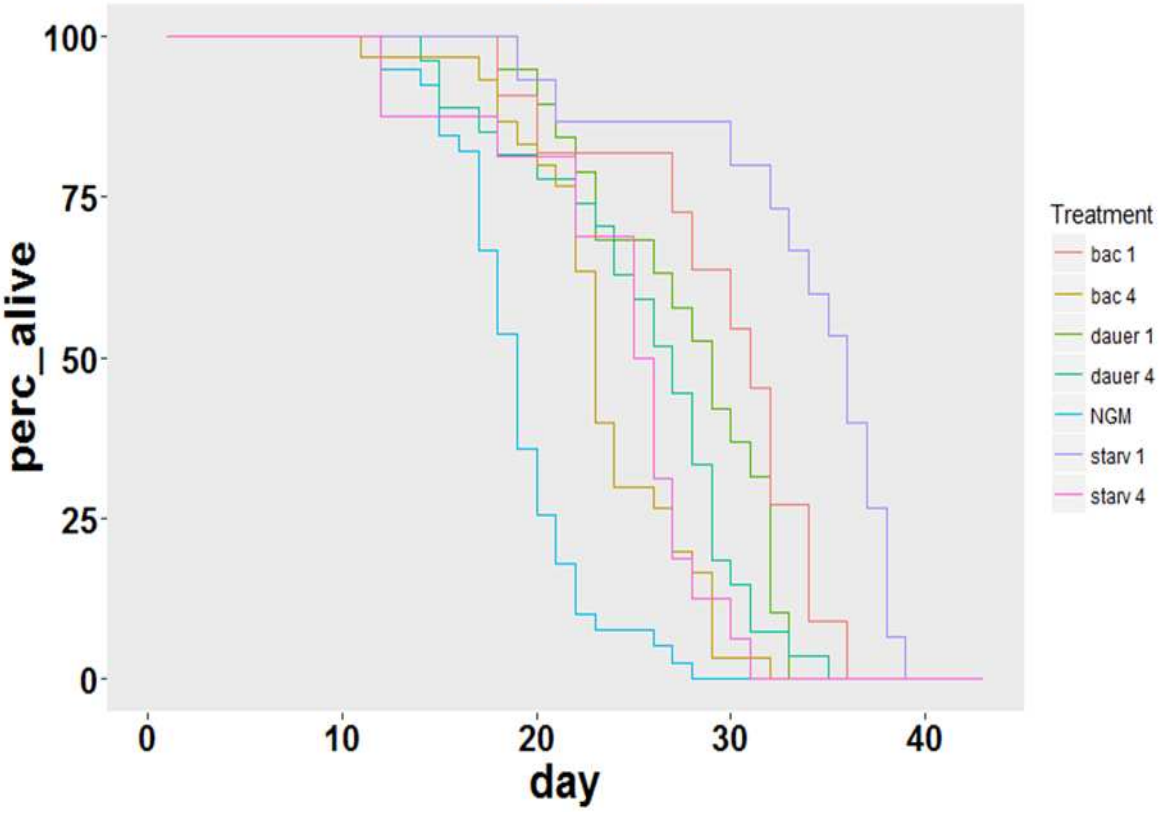


Figure 6.3 Various DR treatments for Line YZ67. Normal lifespan (NGM), peptone withdrawal (dauer), bacterial dilution $5x10^9$ (bac) and bacterial dilution $5x10^8$ (starv) on day 1 and day 4 of adulthood.

Line Z1 was one of the lines where DR significantly increased lifespan in the initial experiment (by 6.1 days; +42.1%; peptone withdrawal: Chapter 4). A similar outcome was observed in this study (Table 6.3, Figure 6.4) for peptone withdrawal treatment (41% increase, Log-Rank p-value <0.001), whilst bacterial dilution further increased lifespan (48%, p-value <0.001) followed by starvation treatment initiated at day one of the adulthood increased lifespan by (38%, p-value <0.001). Initiation of DR at day 4 of adulthood also resulted in extending of lifespan for dauer (23%, p-value <0.001), bacterial dilution (18%, p-value 0.018) and starvation (36%, p-value <0.001) treatments.

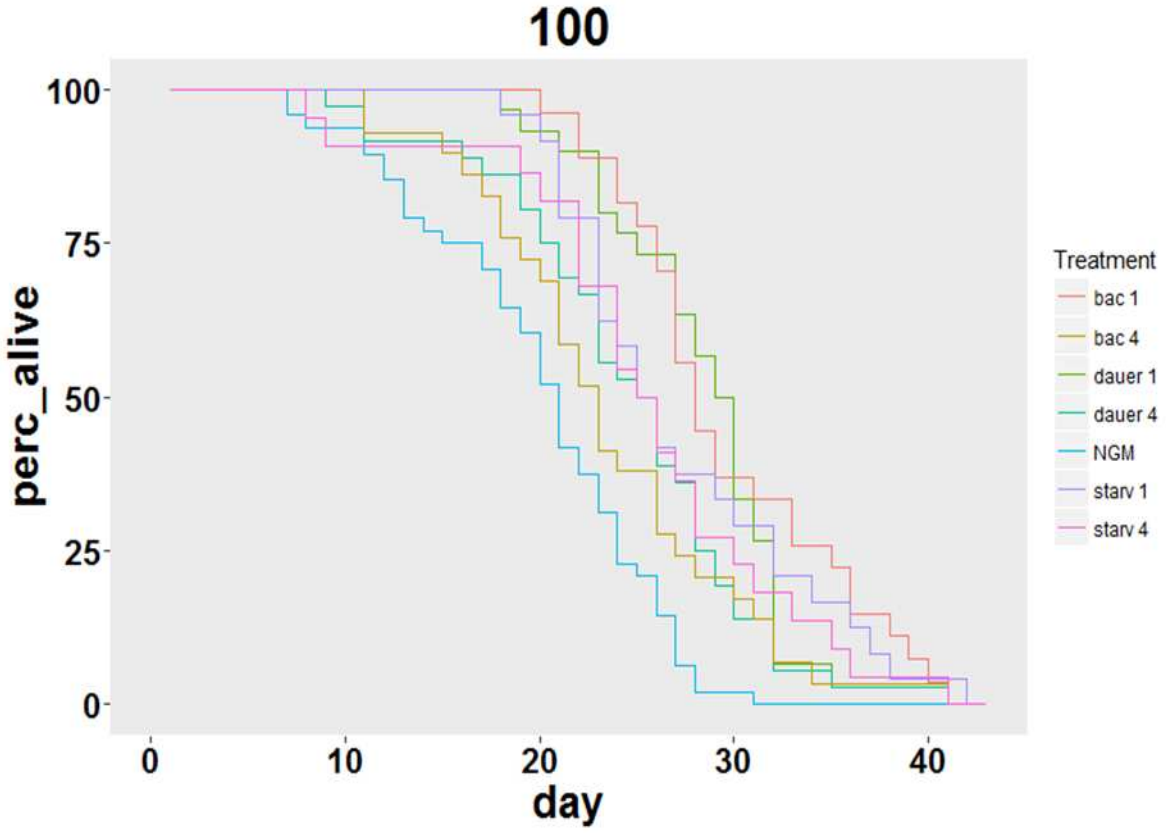


Figure 6.4 Various DR treatments for Line Z1. Normal lifespan (NGM), peptone withdrawal (dauer), bacterial dilution $5x10^9$ (bac) and bacterial dilution $5x10^8$ (starv) on day 1 and day 4 of adulthood.

Line Z11 was originally shorter-lived under mild DR (-24.2%; peptone withdrawal; Chapter 4). This is another line where I have not observed shortening of lifespan under any of the DR conditions (Table 6.3, Figure 6.5). Here, significant life extension was recorded for peptone withdrawal (46%, Log-Rank p-value <0.001), bacterial dilution (73%, p-value <0.001) and starvation (64%, p-value <0.001) treatments initiated at day one of the adulthood. Initiation of DR at day 4 of adulthood also resulted in extending of lifespan for dauer (3%, p-value 0.130), bacterial dilution (43%, p-value <0.001) and starvation (9%, p-value 0.109) treatments. It is very interesting that treatments initiated at day four had, except for the bacterial dilution, the smallest lifespan increase of all treatments. This indicates that this line responds positively to DR only under very specific conditions.

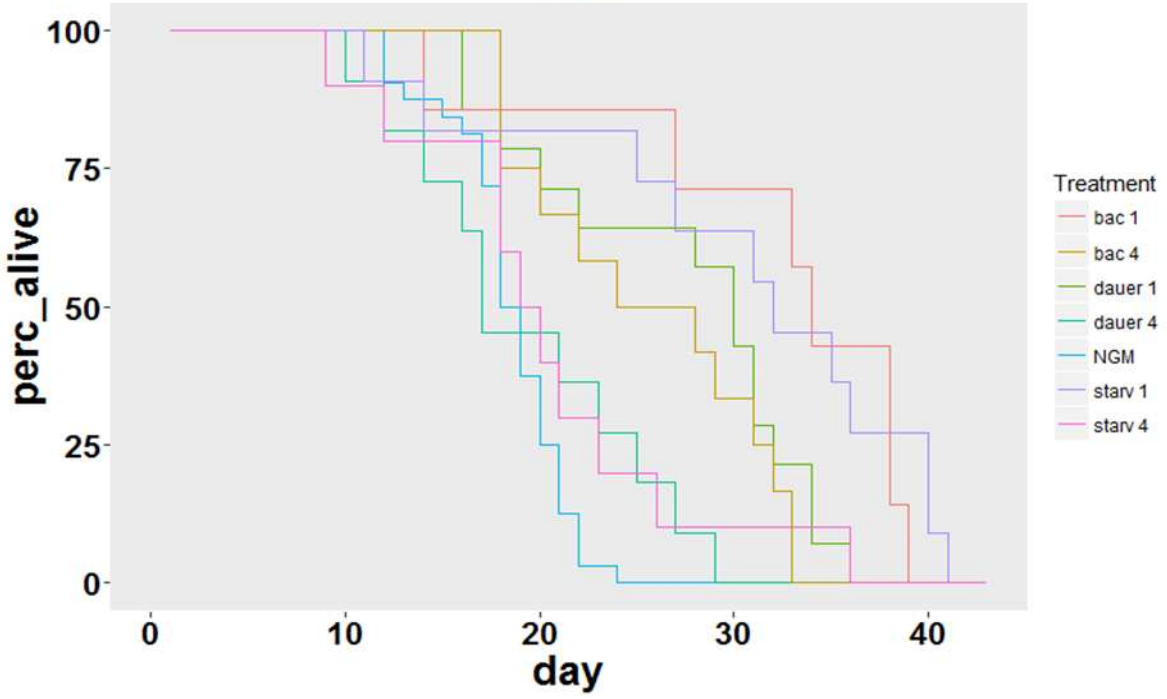


Figure 6.5 Various DR treatments for Line Z11. Normal lifespan (NGM), peptone withdrawal (dauer), bacterial dilution $5x10^9$ (bac) and bacterial dilution $5x10^8$ (starv) on day 1 and day 4 of adulthood.

Line ZY76 was also one of the originally shorter-lived under mild DR (-27%; peptone withdrawal; Chapter 4). As with the other lines, I have not observed shortening of lifespan under any of the DR conditions (Table 6.3, Figure 6.6). Lifespan extension for peptone withdrawal yielded increase of lifespan by (38%, Log-Rank p-value 0.001), bacterial dilution (60%, p-value <0.001) and starvation (49%, p-value <0.001) treatments initiated at day one of the adulthood. Initiation of DR at day 4 of adulthood also resulted in mild lifespan extension for dauer (18%, p-value 0.013), bacterial dilution (23%, p-value <0.001) and starvation (19%, p-value 0.003) treatments. The outcomes are similar to that of line Z11, where the bacterial dilution treatments initiated at both days had the largest life-extending effect.

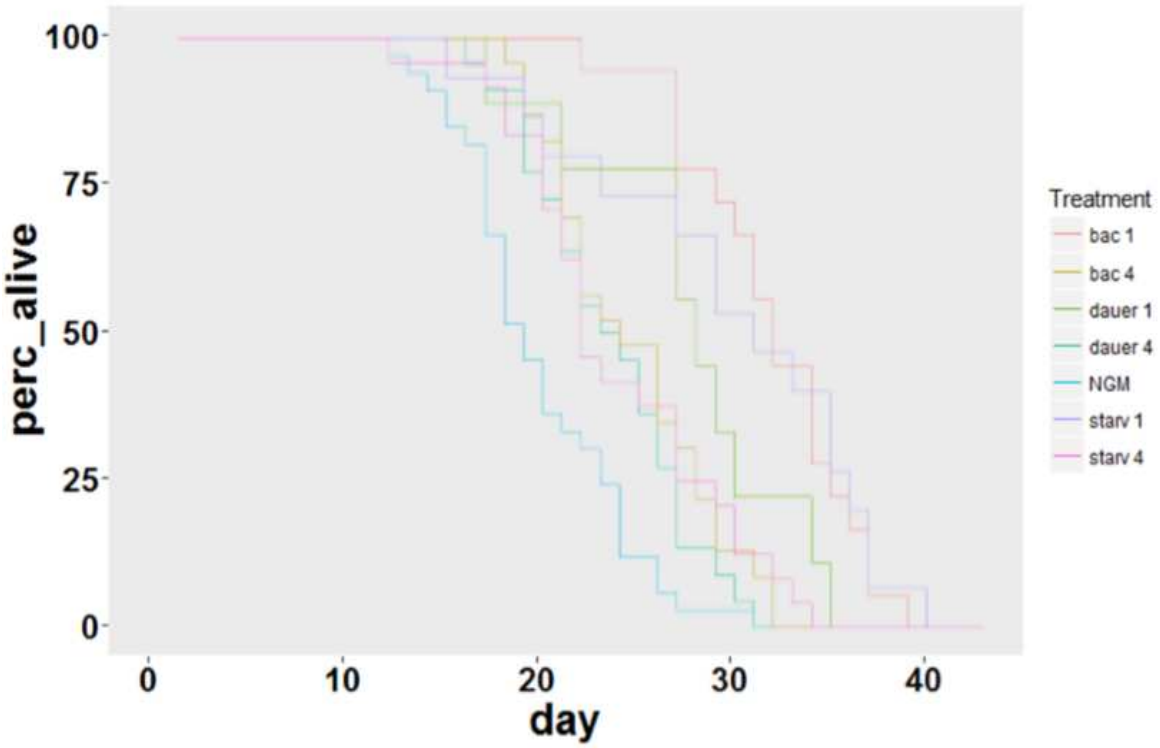


Figure 6.6 Various DR treatments for Line ZY76. Normal lifespan (NGM), peptone withdrawal (dauer), bacterial dilution $5x10^9$ (bac) and bacterial dilution $5x10^8$ (starv) on day 1 and day 4 of adulthood.

N2 was used as a control and to allow for comparison with other DR studies. Lifespan extension was recorded for peptone withdrawal (43%, Log-Rank p-value <0.001), bacterial dilution (62%, p-value <0.001) and starvation (46%, p-value <0.001) treatments initiated at day one of the adulthood (Table 6.3, Figure 6.7). Initiation of DR at day 4 of adulthood also resulted in more moderate lifespan extension for dauer (25%, p-value <0.001), bacterial dilution (32%, p-value <0.001) and starvation (21%, p-value <0.001) treatments. Various DR treatments have increased mean lifespan significantly in all different regimens.

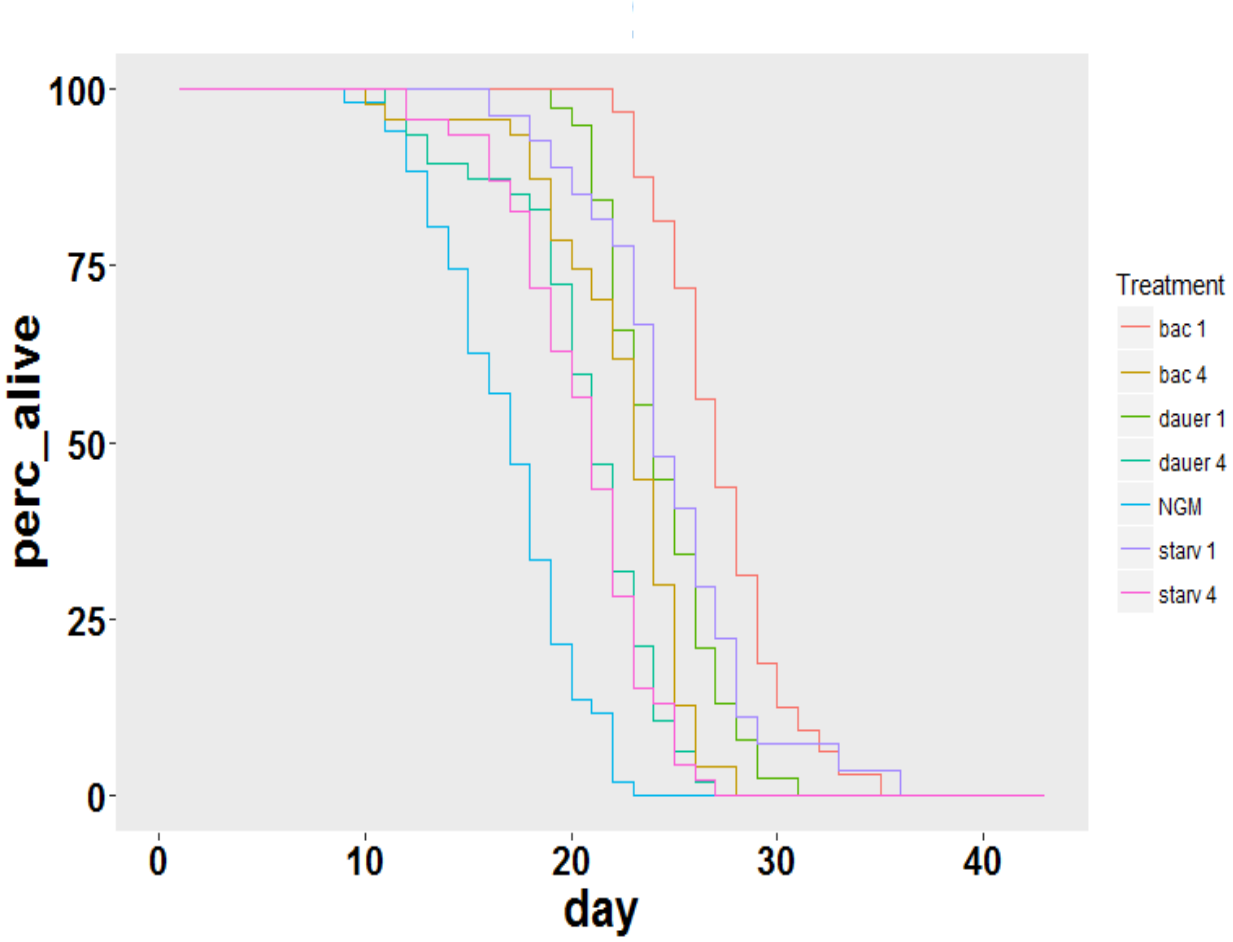


Figure 6.7 Various DR treatments for N2. Normal lifespan (NGM), peptone withdrawal (dauer), bacterial dilution $5x10^9$ (bac) and bacterial dilution $5x10^8$ (starv) on day 1 and day 4 of adulthood.

Overall, DR treated worms appeared morphologically normal under a microscope and have not showed any obvious signs of lethargy. The cases of worms escaping form the petri dish increased with the severity of the treatments. Also increased were the incidences of maternal hatching where, in general, increased maternal hatching was more likely to occur when DR treatment started on day 4 of adulthood.

6.4. Discussion

In *C. elegans*, the longevity response to DR is actively regulated through independent and overlapping pathways (Greer and Brunet, 2009). Previous studies have investigated the effect of multiple methods of DR from mild forms to total starvation (Hosono *et al.,* 1989; Lee *et al.,* 2006; Greer *et al.,* 2006; Smith *et al.,* 2008; Stastna *et al.,* 2015). These studies have reported moderate to extreme lifespan extension in *C. elegans* (13 – 128% increase, Table 6.1). Here, I have performed a side-by-side comparison of various levels and types of DR in *C. elegans*. My results uncover the importance of early implementation of DR regime in nematode worms. In this study, it is also apparent that the lifespan-shortening effects of DR as seen previously in Chapters 2 and 4 were not observed – overall, DR extended lifespan in all lines. This brings an important question, whether the DR shortening of lifespan observed in Chapter 4 on almost third of the RILs is an artefact of small number of worms used in the original experiment. Arguing against this is the fact that the shortening of lifespan by DR was observed in other experiments on various wild isolates and introgression lines of N2/CB4856, which have larger sample sizes. For this reason, it is likely that the effects observed in this study are genotype specific and will need to be explored in greater detail in the future.

The method of bacterial dilution was adapted from Greer *et al*. (2007) with exception of using peptone-free NGM plates as a way of stopping bacterial growth. The initiation of DR at day one and four of adulthood was chosen in order to compare this method to its original paper as well as other DR studies. In general, DR restricted N2 worms from day four of adulthood have similar lifespan extension (5 days) under $5x10^8$ (starvation treatment) bacteria/m as in Greer *et al*. (2007). When DR in N2 was initiated form day one the lifespan extension almost doubled.

In the rest of the lines where DR was initiated from day one of adulthood significant lifespan extension was recorded for peptone withdrawal and bacterial dilution treatments. However, the starvation treatment in most cases except for line YZ67 did not further extended lifespan in most RILs. The response of line YZ67 to starvation treatment was a further increase in lifespan of 82%. This could be an indication that the genetic makeup does play an important part in response to DR.

When DR was initiated at day 4 of adulthood the increase in lifespan was, although still significant in most cases, much lower than the early adulthood initiation. One line showed no significant increase on peptone withdrawal and starvation regimes. These outcomes are not in an agreement with recent Smith *et al*. (2008) study. Here they reported significant life-extension when DR is initiated late in life, even when more than 50% of the population of worms died prior to the initiation of DR. It is clear that the RILs react differently to various environmental challenges.

In the second experiment, total food deprivation regime was used on selected RILs. Here, and consistent with previous results from Lee *et al*. (2006) and Kaeberlein *et al.* (2006), long-term food deprivation leads to increased survival in *C. elegans*. This is in contrast with the more conventional view from other DR studies where too harsh DR treatment fails to prolong

lifespan and/or shorten the lifespan altogether in other organisms such as rats or mice (Barrows and Roeder, 1965). This is likely to be a consequence of the lack of somatic cell division in *C. elegans*, *i.e.* this feature of their development may greatly lower the energy requirement for somatic maintenance. In general, lines that exhibited extended lifespan under earlier DR treatments were more likely to live longer under total starvation.

Further research into various dietary regimens can offer new insights into the ageing processes and the underlining biological mechanisms. With the hope that better understanding of these mechanisms can also provide the means to prevent or delay the age-related diseases in ever ageing and expanding population.

CHAPTER SEVEN: General discussion and future directions

7.1 General Discussion

The increase of human longevity during the last one hundred years is one of the biggest achievements of modern medicine and technology. Postponed senescence now needs to be coupled with decrease in age-related diseases such cardiovascular diseases, dementia and other chronic diseases in order to avoid huge economic and social costs in near future. An improved understanding of biology of ageing can assist in this by introducing novel solutions for dealing with the growing older population. One of the approaches to the study of ageing, and a potential intervention that might allow lifespan extension in humans whilst also improving health, is the use of DR. For almost a century DR approach appears to be the most robust and reliable method of postponing senescence and delaying age-related diseases (Mair and Dillin, 2008; Gredilla and Barja, 2005). There are, however, many unanswered questions about the mechanisms and efficiency of DR. Research on the mechanisms of ageing in model organisms has made a crucial contribution to the study of ageing and to our developing understanding of DR – but again there remains more that can be done.

In this thesis, I have used the model nematode *C. elegans*, with its short lifespan, high fecundity and wide array of genetic and genomic resources, to investigate variation in lifespan, in traits related to lifespan and in the response to DR. My specific aims were to further characterise a new panel of 4-parent RILs. C. elegans has been extensively used in unravelling the intricate details of the ageing phenotype. For example, it was experiments in this model system that uncovered the importance of nutrient signalling pathways in ageing (Kenyon et al., 1993). The current approach in most studies using *C. elegans* is however to use a single genetic

background, and although this is informative about the molecular bases of ageing, this approach does not tell us about natural populations or allow any understanding of the effects of variation between worm isolates on the complex traits we are interested in. My approach has therefore been to identify natural variation and, where possible, to avoid situations where the extensive lab adaptation seen in N2 may confound results.

My analysis of lifespan QTLs detected in N2/CB4856 ILs (Doroszuk *et al*., 2009) replicated most of the previously identified QTLs, but suggests that three of these QTLs are potentially the result of incompatibilities between the N2 and CB4856 genomes. This is the case because these three QTLs are associated with greatly increased rates of maternal hatching – a synthetic egg-laying defective phenotype that is not seen in CB4856 and N2 nor in other wild isolates (Snoek *et al.,* 2014). QTL mapping of maternal hatching in N2/CB4856 recombinant inbred lines (RILs) recovers one of the most extreme of the maternal hatching QTLs, again supporting the idea that phenotypes associated with this region represent negative epistatic interactions between alleles from the different parents rather than alleles that affect the trait within their 'normal' genetic background.

Maternal hatching in *C. elegans* is also associated with ageing, with the rate increasing with age over the reproductive period. This work also uncovers variation between ILs and also between wild isolates in the effect of DR on lifespan (Chapter 2/Stastna *et al.,* 2015) and, in many strains, an increased rate of maternal hatching in response to DR. This alteration in the rate of maternal hatching in response to DR may represent the effects of stress or damage – which in some of the ILs may be aggravating the effects of the incompatibility – or may be an adaptive parental response to starvation (Chen and Caswell-Chen, 2004; Chen and Caswell-Chen, 2003).

In combination, these analyses of N2/CB4856 ILs and RILs and the associated work on a small group of wild isolates are important because they highlight the importance of considering synthetic interactions between genetic backgrounds when undertaking QTL mapping. This research is also important as it is the first to show that DR in *C. elegans* does not always prolong lifespan *i.e.* that the effect on lifespan depends on the genetic background.

The finding that DR in *C. elegans* affects lifespan in a genotype-specific manner, and that in some genotypes DR reduces lifespan, led me to consider the effects of DR more broadly. Consideration of the DR literature revealing that there are many studies from a wide range of taxonomic groups where various DR treatments had no effect or a lifespan shortening effect (as reviewed in Chapter 3). This questions the dogma of DR as a universal means of lifespan extension. Either DR is not in fact a universal way of doing this or there must be specific methodological reasons why these studies failed to show positive effects of DR. Review of these studies did identify cases where the, some of these outcomes are likely to be due to harsh DR regimens, small sample sizes or even unsuitable diets. However, this still does not explain the scale of research where various treatments failed to prolong lifespans in a wide range of taxa. This at least partially supports the idea that varied responses to DR treatments are due to biological differences and that DR can be deleterious for some genotypes. However, DR analyses carried out later in this study do argue against this.

Given that nearly all research in *C. elegans* biology has been done on N2 or it derived mutants and work on the previously isolated QTLs highlights the issue of incompatibilities between CB4856 and N2 (Chapter 2), there is clearly a need for research that can explore the full potential of the natural genetic variation in wild-derived populations. To allow for this, a novel 4-parent panel of 200 sequenced and genotyped RILs was analysed (Chapters 4-7). This panel

was derived from freshly isolated wild strains of *C. elegans* from two geologically separate locations in France. This means that the panel will not contain any of the alleles underlying the extensive lab adaptation seen in N2 (Sterken *et al.,* 2015) and hence variation is likely to be ecologically relevant. Many previous studies have investigated *C. elegans* response to various environmental factors in a limited number of genetic backgrounds (Harvey and Viney, 2007; Vinuela *et al.* 2011; Sinha et al. 2012; Gidalevitz *et al.* 2013). In Chapter 4, I analysed the novel multi-parent RILs for response to lifespan under normal conditions and under mild DR conditions and for survival in response to heat shock, cold shock and oxidative stress. The response from the panel was highly variable in all treatments indicating that the four recent wild isolates used to construct the RILs contain multiple alleles affecting these traits. Interestingly, and contrary to expectations, only the two lifespan traits showed any correlation between the RILs. Given that stress and lifespan are closely related and a successful strategy for isolating mutations affecting lifespan in *C. elegans* has been to screen for increased stress resistance (see Johnson *et al.,* 2002 for review), detecting no correlation between stress resistance traits and lifespan was an interesting outcome. This may imply that the types of mutations that can be produced in the lab, and that affect both lifespan and stress resistance, are qualitatively different from the sorts of mutations that are maintained in the wild.

As in the analysis of the N2/CB4856 ILs and the wild isolates (Chapter 2), a mild DR treatment seemed to have life-shortening effect on some of the RILs. One possible explanation for this observation is that the wild populations within the natural environment are often exposed to longer periods of less extreme environmental changes and therefore possess the ability to withstand such conditions and the response to the various stressors differs among the phenotypes (Li *et al.,* 2006). Also a possible reason why this has not been observed before may

be the fact that prolonged laboratory conditions can lead to trade-offs between lifespan and some stress resistance (Reznick and Ghalambor, 2005).

To uncover the genetic factors lie behind the extensive variation observed in the RILs, a quantitative genetic approach was taken (Chapter 5). This resulted in 20 potential QTLs for the various traits. Some of the QTLs that appeared to co-locate, especially in a large region of chromosome lll. This may suggest that, despite the lack of correlations between traits, there may be some pleiotropic variants in this region. Comparison of these results to previous studies indicates that although some of the QTL regions found in this study have been previously detected, others have not. For example, no QTL on chromosome V was detected, despite previous reports by other studies of variation in lifespan and stress resistance mapping to the region (Ebert *et al*., 1993; Ayyadevara *et al*., 2001; Ayyadevara *et al*., 2003; Doroszuk *et al*., 2009). This is an interesting outcome and highlights the value of using different genetic backgrounds and of using RILs not constructed using the canonical strain N2.

Comparing the genes underlying the QTLs identified here to the known genes that to regulate lifespan in *C. elegans*, particularly those from the various nutrient-sensing pathways, indicates that many QTLs do contain such genes. This does not mean that these genes are responsible for the effects seen here, only that they could. Importantly though, this comparison identifies several of my QTLs that do not contain any of these key aging-related genes and therefore cannot be explained by explained by these known regulators. This indicates that the approach of consideration variation in genetic backgrounds other than N2 is sound in that it has allowed detection of effects that are a consequence of novel regulators of ageing. The identification of the underlying polymorphisms for these QTLs is therefore a priority for future work. Given that one of my QTLs also maps to a very small region, containing just 17 genes, identifying the causal

variant underlying this QTL is also an attractive proposition. In addition to these specific future goals, this work has also served an important function in validating the role of these 4-parent RILs in providing a novel and versatile resource for quantitative genetics that has been notably missing in *C. elegans*.

Of the QTLs identified for survival in response to stress, the most striking was the cold stress resistance QTL on chromosome III (Chapter 5). However, no genes known to be involved in the *C. elegans* cold stress response were found within this QTL region. Interestingly, the various studies on cold shock survival in *C. elegans* have tested for different outcomes. For example, several studies assessed mortality after 60 min of recovery at the growth temperature, with worms scored as dead if they did not respond to touch or show pharyngeal pumping (Iser et al., 2005; Murray et al., 2007; Otha et al., 2014), whilst Savory et al. (2011) monitored survival after 20-30 minutes at the room temperature. As there is significant mortality in the 24 hours after return to growth temperature (Robinson and Powell, 2016), it is not clear what the effect on longer-term survival would have been in studies with a shorter recovery period. Robinson and Powel (2016) postulate that there are two distinct phases of death in worms in response to cold shock. The first of these occurs during, or straight after, the treatment as a result of cellular damage and the second occurring as the worms try to deal with aftermath of cold shock. This is partially supported by study of the onion maggot *D. Antigua*, where, after exposure to cold treatment the maggot displays two different type of deaths by morphology (Clark and Worland, 2008). My analyses, which are based on a 24-hour recovery period, identify a broad range of responses to cold stress in *C. elegans* wild isolates and indicate that translation regulation is critical to cold stress survival in *C. elegans.* Further analysis of the chromosome III region I identify suggests that the QTL may be a consequence of variation in

eftu-2 (a homologue of human eEF2) and part of the translation machinery. This is supported by eQTL results from the 4-parent RILs and also from studies on N2/CB4856 RILs. Functional testing of this is therefore a priority for future work. Finally, although, the mechanisms of cold tolerance are interesting for many reasons such as the potential for use in medicine, they are also interesting in their own right. Particularly as not much is understood about the ecology of cold stress resistance, there is definitely future value in combining such studies with those on genome plasticity and evolution.

Given, the varied outcomes of dietary restriction (DR) in previous chapters, several different approaches were adopted in Chapter 7 to uncover to what extent DR treatment prolongs lifespan depending on initiation date and the severity of the treatment. My analyses uncovered that an earlier initiation day (starting from the adulthood) yields a larger lifespan-extension. When DR was initiated at a later date, the increase in lifespan was, although still significant in most cases, much lower than the early adulthood initiation. This is in contrary to the results of Smith *et al*. (2008) who reported a greater increase in lifespan in worms submitted to DR at older ages. Also the severity of the DR seems to bring up different responses among the selected RILs. This again indicates that there are genotype-specific responses to various environmental challenges. Total starvation of the worms resulted in lifespan extension in most of the lines as previously reported by Lee *et al*. (2006) and Kaeberlein *et al.* (2006). In general, lines that exhibited extended lifespan under earlier DR treatments were more likely to live longer under total starvation.

Overall, this study shows that the novel multi-parent panel provides a versatile resource toward easier and efficient fine mapping and functional analyses of loci and genes underlying complex traits which can accelerate the discovery of natural polymorphisms underlying

complex traits and will lead to a better understanding of the mechanisms behind the observed phenotypic variation in *C. elegans.*

7.2 Principal findings

In summary, the key findings of this thesis have been:

- That the effect of dietary restriction on lifespan in *C. elegans* varies between genotypes and that such differences are seen in introgression lines, recombinant inbred lines and in wild isolates.
- That the literature on DR supports the view that genotype-specific effects on lifespan are widespread and that for some genotypes DR can be deleterious.
- However, some of the later results in various DR regimes were conflicting with the previous results.
- That a newly created 4-parental panel of RILs contains significant, ecologically relevant variation in lifespan and stress resistance and that lifespan and stress resistance are not correlated in these lines.
- This can be used to identify quantitative trait loci (QTLs) controlling this variation.
- That multiple candidate QTLs have been identified for lifespan and stress resistance and that some of these QTLs cannot be explained by known lifespan regulating genes.
- That cold stress resistance in *C. elegans* is related to the control of translation, that the major QTLs detected in the RILs cannot be a consequence of genes known to be involved in cold stress resistance and that one may be a consequence of variation in *eftu-2* a part of the translation machinery.

7.3 Future directions

Given the key findings of the work reported in this thesis, the immediate priorities for future work are to:

- Extend and replicate the studies on dietary restriction to uncover the reasons why the effects of DR vary across genotypes.
- Identify the causal polymorphisms underlying the lifespan QTLs that cannot be explained by known lifespan-regulating genes and determine how the gene(s) identified integrate into the pathways that determine lifespan in *C. elegans*.
- Identify the causal polymorphisms underlying the cold stress resistance QTLs in *C. elegans* and determine how this resistance is produced.

Over the longer term, the work I have undertaken here should be extended by creating of introgression lines from the parental backgrounds. Furthermore, the hope is that the 4-parental RILs will be shared and used on wide array of experiments to explore their full potential. In addition, I hope that future DR research will take into consideration the importance of genetic backgrounds.

7.4 Activities arising from this thesis

7.4.1 Publications:

Stastna J.J., Snoek L.B., Kammenga J.E. & Harvey S.C. (2015) Genotype-dependent effects of dietary restriction on lifespan in *Caenorhabditis elegans*. Scientific Reports 5, 16259.

7.4.2 Manuscript in preparation:

Jana J Stastna, L Basten Snoek, Nell Nei, Yiru Wang, Joost AG Riksen, Jan E Kammenga, Simon C Harvey. The molecular basis of natural variation in cold stress response in Caenorhabditis elegans

Jana J. Stastna & Simon C. Harvey. Dietary restriction is not a universal mean of postponing senescence

7.4.2 Presentations:

Stastna J.J., Snoek L.B., Riksen J.A.G., Vujakovic A., Schulenburg H., Kammenga J.E. & Harvey S.C. (2016) Natural variation in stress response in the Nematode Caenorhabditis elegans using a novel multi-parent recombinant inbred line panel. **Talk**. BSRA Meeting, Dutham, 4th – 6th July

Stastna J.J., Snoek L.B., Riksen J.A.G., Vujakovic A., Schulenburg H., Kammenga J.E. & Harvey S.C. (2016) Natural Variation in Acute stress resistance, DR and lifespan in a multi-parent recombinant inbred line panel in C. elegans. **Poster presentation**. European Worm Meeting, Berlin, 1st – 4th June

Stastna J.J., Snoek L.B., Volkers R.J.M., Riksen J.A.G., Vujakovic A., Schulenburg H., Kammenga J.E. & Harvey S.C. (2015) Acute stress resistance, DR and lifespan in a multi-parent recombinant inbred line panel. **Poster presentation**. C. elegans International meeting, UCLA, Los Angeles 24th – 28th June

Stastna J.J., Snoek L.B., Kammenga J.E. & Harvey S.C. (2015) Dietary restriction is not a universal means of prolonging the lifespan in nematode Caenorhabditis elegans. **Poster presentation**. Healthy Ageing: From Molecules to Organisms, Cambridge, 18th – 20th May.

Stastna J.J., Snoek L.B., Volkers R.J.M., Riksen J.A.G., Schulenburg H., Kammenga J.E. & Harvey S.C. (2014) Natural variation in lifespan and dietary restriction effects in a multi-parent recombinant inbred line panel in Caenorhabditis elegans. **Poster presentation**. Evolutionary Biology of Caenorhabditis and other Nematodes, Cambridge, 14th-17th June.

Stastna J.J., Snoek, L.B., Volkers R.J.M., Riksen J.A.G., Kammenga J.E., Harvey S.C. (2014) Lifespan and the effects of dietary restriction in a multi-parent recombinant inbred line panel in Caenorhabditis elegans. **Poster presentation**. European C. elegans Meeting, Berlin, 15th – 17th May.

7.4.3 Research awards:

Research Prize Winner at the Wellcome Trust Conference 2015: Healthy Ageing: From Molecules to Organisms (Held on 18-20 May 2015, Cambridge, UK.

APPENDIX A

Line	Lifespan	DR Lifespan
Y10	17.71	20.5
Y11	17.5	17.5
Y12	13.09	14.083
Y13	17.938	18.18
Y14	17.625	17.3
Y15		
Y16	26.27	27.294
Y17	17.83	21.6
Y18	19.6	19.6
Y19	15.5	15.625
Y2	20.7	19.286
Y20	18.75	21
Y21	21.3	15
Y22	19.7	21.3
Y23	20.2	18.4
Y24		
Y25	16.81	21.6
Y26	14.83	20.16
Y27	20.57	23.18
Y3	17.18	18.375
Y5	19.54	18.3
Y6	20.05	22.8
Y7	20.875	21.875
Y8	17	17.625
Y9	17.14	19.25
YZ1	15.6	18.125
YZ10	18.16	19.462
YZ11	16.857	16.286
YZ12	20.71	23.29
YZ13	22	21.824
YZ14	13.16	16.375
YZ15	18.083	16.571
YZ16	19.1	20.929
YZ18	18.63	13
YZ19		
YZ2	13.625	17.14
YZ20	17.143	16.416
YZ21		
YZ22	21.2	21.4
YZ23	19.4	19.63

Line	Lifespan	DR Lifespan
YZ24	19.1	17.5
YZ25	16.6	17.333
YZ26	18.625	19.46
YZ27	15.92	20
YZ28	17.8	18.214
YZ29	17.8	17.625
YZ3	21.1	23.6
YZ30	17.4	14.6
YZ31	15.429	17.857
YZ32	13.6	19.57
YZ33	17.57	19.556
YZ34	17.0625	16.4
YZ35	18.94	23.45
YZ36	18.4	16.4
YZ37	17.23	16.143
YZ38	19.5	17.7
YZ39	13.72	20.4
YZ4	21.2	20
YZ40	18.916	18
YZ41	15.176	17.526
YZ42	19.16	20.2
YZ43	17.3	17.75
YZ44	18.06	17.909
YZ45	17.375	23.286
YZ46	15.3	17.625
YZ47	16	18.25
YZ48	17.875	15.3
YZ49	14.75	18.3
YZ5	22.4	21.09
YZ50	13	16.6
YZ51	20.428	18
YZ52	15.75	19.375
YZ53	15.75	17.143
YZ54	15.16	18.4
YZ55	17.125	16.765
YZ56	15	17.25
YZ57	17.4	15.8
YZ58	18.13	18.83
YZ59	17.625	21
YZ6	16.1	19.75

Line	Lifespan	DR Lifespan
YZ60	15.571	16.571
YZ61	16.6	21.25
YZ62	23.3	23
YZ63	15.57	16.8
YZ64	16.4	16.63
YZ65	16.2	14.846
YZ66	18.5	18.7
YZ67	21	17
YZ68	14.9	16.3
YZ69	12.72	19.615
YZ7	19	24
YZ70	20	18.83
YZ71	14.5	15.5
YZ72	18.63	22.75
YZ73	19.25	21.375
YZ74	15.94	19.083
YZ75	17.75	19.357
YZ8	17.7	20.8
YZ9	19.647	19.364
Z1	14.5	20.6
Z10	15.75	17
Z11	20.625	15.625
Z12	20.571	20.429
Z13	18.5	19
Z14	20.4	17.75
Z15	15.357	18.5
Z16	15.09	16.5
Z18	15.69	14.3
Z2	15.09	18.1
Z20	18.538	16.625
Z21	18.875	19.882
Z22	17.16	16
Z23	20.25	20.8
Z24	15.0625	18.3
Z25	18.18	18.13
Z26	19.667	15.875
Z27	16.563	16.571
Z3	16.6	19.214
Z30	17.286	17.4
Z4	17.72	22.5
Z5	16.56	13.75
Z6	15.4	20.75
Z7	16.3	19.2

Line	Lifespan	DR Lifespan
Z8	23.4	19.7
ZY1	20.8	19.4
ZY10	14.1	18.25
ZY11	16.6	18.5
ZY12	18.625	20.615
ZY13	17.6	20.923
ZY15	21.22	24.25
ZY16	20.474	24.714
ZY17	22.36	18.286
ZY18	19.2	21.5
ZY19	17.25	18.2
ZY2	20.786	17.364
ZY20	18.125	23.25
ZY21	20.476	20.5
ZY22	15.2	19.54
ZY23	15.45	18.25
ZY24	19.6	20.5
ZY25	21.091	21.375
ZY26	15.3	17.416
ZY27	21.916	20.46
ZY28	19.27	22.375
ZY29	18.47	22.929
ZY3	18.286	23
ZY30	20.06	22.143
ZY31	21.186	21
ZY33	13.538	20.5
ZY34	20.125	21.3
ZY35	22.571	23.54
ZY36	16.71	20.8
ZY37	21.286	21.27
ZY38	17.09	19
ZY39	16.06	21
ZY4	19.857	21.14
ZY40	22.93	20.429
ZY41	17.57	17.625
ZY42	20	24.3
ZY43	19	18.4
ZY44	14.57	20.636
ZY45	19.83	23
ZY46	19.53	23.8
ZY5	19.75	19.857
ZY51	22	21.09
ZY52	20.421	24.615

RILs	Lifespan	DR lifespan
ZY53	17.1	20.571
ZY54	17.72	16.462
ZY55	16.57	17.6
ZY56	21.214	19.5
ZY58	19	20.444
ZY59	19.57	20.333
ZY6	15.571	14.571
ZY60	16.1	20.625
ZY61	22.85	20.2
ZY62	15.12	22.083
ZY63	17.6	19.5
ZY64	18.429	18.909
ZY65	19.3	20.875
ZY66	21	20.643
ZY67	22.54	22.1
ZY68	18.416	18.6
ZY69	17.26	18.93
ZY7	19	16.3
ZY70	21.45	25.75
ZY71	16.5	18.588
ZY72	19.7	16.6
ZY75	18.6	21
ZY76	20	14.6
ZY77	19.75	25.16
ZY78	18.5	21
ZY79	17.385	21
ZY8	16.4	20.286
ZY80	19.136	23.235
ZY81	21.3	25.375
ZY82	22.27	22.81
ZY83	18.125	17.727
ZY91	20.36	24.8

Table A1. All RILs lines used in this study.

This table represents all RILs and their average lifespan for *ad libitum* and DR treatments

APPENDIX B

```
#clear memory
rm(list=ls())
#switch working directory and confirm
getwd()

#load data
lifespan.dat <- read.csv("lifespan1.csv")

#define function to simulate sampling a number of times (x) from a normal distribution of
defined mean (y) and standard deviation (z), repeating the sampling for a different set of
conditions and then calculating the difference between them
#means of the distributions set to the global mean of the RIL data, 18.138 NGM and 19.419
DR, meaning the effect of DR is a 1.281 day extension of lifespan
delta <- function(x,y,z) {
  mean(rnorm(x, y+1.281, z)) - mean(rnorm(x, y, z))
}

#simulate data assuming various levels of sample size per treatment
data5 <- replicate(1000, delta(5,18.138,3))
ranked5 <- sort(data5)

data10 <- replicate(1000, delta(10,18.138,3))
ranked10 <- sort(data10)

data20 <- replicate(1000, delta(20,18.138,3))
ranked20 <- sort(data20)

#rank data from the RILs
diff1 <- lifespan.dat$diff
diff <- sort(diff1, decreasing=F)

#plot figure
plot (ranked5, xlab='', ylab='Difference in lifespan', ylim=c(-8,8), col="red")
par(new=T)
plot (ranked10, xlab='', ylab='', ylim=c(-10,10), axes=F, col="sandybrown")
par(new=T)
plot (ranked20, xlab='', ylab='', ylim=c(-10,10), axes=F, col="peachpuff")
par(new=T)
plot (diff, xlab='', ylab='', ylim=c(-10,10), axes=F, col="black")
abline(h=1,col=4,lty=2)
abline(h=0)
```

APPENDIX C

Gene	Overview
aap-1	***aap-1*** negatively regulates lifespan and dauer development, and likely functions as the sole adaptor subunit for the AGE-1/p110 PI3K catalytic subunit to which it binds in vitro; although AAP-1 potentiates insulin-like signaling, it is not absolutely required for insulin-like signaling under most conditions.
akk-1	***aak-1*** activity is required, in parallel with aak-2 and downstream of *daf-2, daf-7,* and *par-4,* for negative regulation of germline proliferation during dauer development.
aak-2	***aak-2*** functions downstream of environmental stressors, energy level signals (AMP:ATP ratio), and *daf-2*-mediated insulin signaling to positively regulate adult lifespan; in regulating lifespan, ***aak-2*** likely acts in parallel with *daf-16*/FOXO; in the germline,aak-2 functions downstream of *daf-2* and *daf-7,* and in parallel to *par-4* and *aak-1*, to negatively regulate germline proliferation during dauer development.
akt-2	**akt-2** encodes a homolog of the serine/threonine kinase Akt/PKB, AKT-2, that is required for progression through the dauer stage of development and for the negative regulation of adult lifespan; inactivation of *akt-2* causes animals to arrest constitutively at the dauer stage, while having an increased life span; widely expressed, AKT-2 is activated by the phospholipid products of phosphoinositide 3-kinase AGE-1/PI3K and by PDK-1, a homolog of vertebrate 3-phosphoinositide-dependent kinase-1 (PDK-1) Normal *akt-2* (and *akt-1*) activity is required for excess *pdk-1* activity to suppress the dauer-arrest phenotype of *age-1*, indicating that the 3-phosphoinositide-dependent kinase-1 homolog PDK-1 transduces signals from AGE-1 to AKT-2 (and AKT-1); conversely, the *akt-2* loss-of-function phenotype is suppressed by daf-16 null mutations, indicating that the Fork head transcription factor DAF-16 is downstream of AKT-2 (and AKT-1), and that AKT-1 and AKT-2 act primarily to antagonize DAF-16.
ctl-1	***ctl-1*** encodes one of three *C. elegans* catalases; CTL-1 exhibits catalase activity in vitro, and thus likely functions in vivo as an antioxidant enzyme that protects cells from reactive oxygen species; ***ctl-1*** activity contributes to the extended lifespan seen in *daf-2* mutant animals; in addition, ***ctl-1*** expression is negatively regulated by DAF-2-mediated insulin signalling.
ctl-2	***ctl-2*** encodes one of three *C. elegans* catalases; CTL-2 exhibits catalase and peroxidase activity in vitro, and thus likely functions in vivo as an antioxidant enzyme that protects cells from reactive oxygen species; ***ctl-2*** activity is required for normal lifespan as well as for the extended lifespan seen in *daf-2* mutant animals; ***ctl-2*** expression is negatively regulated by DAF-2-mediated insulin signaling.
ctl-3	***ctl-3*** encodes one of three *C. elegans* catalases; CTL-3 is predicted to function as an antioxidant enzyme that protects cells from reactive oxygen species; a ***ctl-3*** promoter gfp fusion construct is expressed in pharyngeal muscles and neuronal cell bodies; loss of ***ctl-3*** activity via RNAi results in no obvious abnormalities.

cst-1	***cst-1*** encodes one of two *C. elegans* protein kinases orthologous to the MST (mammalian Ste20-like) kinases and *Drosophila* Hippo; CST-1 appears to play a role in the responses to oxidative stress and determination of adult lifespan; in regulating stress response and lifespan, CST-1 functions upstream of the DAF-16/FOXO transcription factor; CST-1 physically interacts with RSF-1, the *C. elegans* homolog of the Ras-association domain family protein 1.
C10H11.8	**C10H11.8** by homology, the product of C10H11.8 is predicted to function as a member of the TOR complexes to regulate growth, development, and actin cytoskeleton organization; loss of C10H11.8 activity via RNAi results in increased fat content, developmental delays, and small body size; RNAi targeting C10H11.8 as well as *let-502* results in defects in P cell migration.
ddl-3	***ddl-3*** is an ortholog of human TTC19 (tetratricopeptide repeat domain 19); ***ddl-3*** is involved in determination of adult lifespan.
daf-2	***daf-2*** encodes a receptor tyrosine kinase that is the *C. elegans* insulin/IGF receptor ortholog; DAF-2 activity is required for a number of processes in *C. elegans*, including embryonic and larval development, formation of the developmentally arrested dauer larval stage (diapause), larval developmental timing, adult longevity, reproduction, fat storage, salt chemotaxis learning, and stress resistance, including response to high temperature, oxidative stress, and bacterial infection; DAF-2 signals through a conserved PI 3-kinase pathway to negatively regulate the activity of DAF-16, a Forkhead-related transcription factor, by inducing its phosphorylation and nuclear exclusion; in addition, DAF-2 negatively regulates the nuclear localization, and hence transcriptional activity, of SKN-1 in intestinal nuclei; amongst the 38 predicted insulin-like molecules in *C. elegans*, genetic and microarray analyses suggest that at least DAF-28, INS-1, and INS-7 are likely DAF-2 ligands; genetic mosaic and tissue-specific promoter studies indicate that ***daf-2*** can function cell nonautonomously and within multiple cell types to influence dauer formation and adult lifespan, likely by regulating the production of secondary endocrine signals that coordinate growth and longevity throughout the animal; temporal analysis of ***daf-2*** function indicates that ***daf-2*** regulates lifespan, reproduction, and diapause independently, at distinct times during the animal's life cycle.
daf-3	***daf-3*** functions as a transcriptional regulator that is required for formation of the alternative dauer larval stage as well as for regulation of pharyngeal gene expression during non-dauer development; DAF-3 activity is antagonized by signaling through the DAF-7/TGF-beta pathway which promotes reproductive growth; in yeast two-hybrid studies, DAF-3 interacts with SMA-3, another Smad protein that does not appear to have a role in dauer formation;.
daf-4	***daf-4*** activity is required for several biological processes, including entry into and exit from the dauer larval stage, body size determination, male tail patterning, egg laying, chemosensory neuron specification, and increased thermotolerance; in regulating dauer larval development, DAF-4 acts in parallel with the insulin signaling pathway and likely partners with the DAF-1 type I TGF-b receptor to receive a signal from the DAF-7 TGF-b ligand; ***daf-4*** also regulates reproductive aging, via the TGF-beta Sma/Mab pathway, mutants of which show a large reproductive span extension.

daf-7	***daf-7*** functions as part of a signalling pathway that interprets environmental conditions to regulate energy-balance pathways that affect dauer larval formation, fat metabolism, egg laying, pathogen avoidance behavior, and feeding behavior.
daf-9	***daf-9*** likely functions cell nonautonomously in hypodermal and neuronal cells to produce, for the DAF-12 nuclear receptor, a lipophilic hormone whose presence is necessary for bypassing entry into the alternative L3/dauer larval stage and promoting reproductive development; in regulating dauer formation,daf-9 acts downstream of the DAF-2/insulin/IGF receptor and the DAF-7/TGFbeta ligand, suggesting that at least two of the signaling pathways that control dauer formation converge, in part, upon ***daf-9***; in addition, ***daf-9*** activity is required for gonadal cell migration.
daf-12	***daf-12*** affects dauer formation downstream of the TGF- and insulin signaling pathways, and affects gonad-dependent adult longevity together with DAF-16, chemosensory signal transduction cells; ***daf-12*** expression in lateral seam cells is negatively regulated by the let-7 miRNA.
daf-16	***daf-16*** encodes the sole C. elegans forkhead box O (FOXO) homologue; DAF-16 functions as a transcription factor that acts in the insulin/IGF-1-mediated signaling (IIS) pathway that regulates dauer formation, longevity, fat metabolism, stress response, and innate immunity; DAF-16 can interact with the CBP-1 transcription cofactor in vitro, and interacts genetically with other genes in the insulin signaling and with ***daf-12***, which encodes a nuclear hormone receptor.
fkb-2	***fkb-2*** by homology, FKB-2 could function in a number of processes including protein folding, signal transduction, and regulation of muscle contraction; however, the precise role of FKB-2 in *C. elegans* development and/or behavior is not yet known,.
ftt-2	***ftt-2*** encodes a 14-3-3 protein; FTT-2 is required for regulating the localization of the product of YAP-1, a Yes-associated protein (Yap) homolog, between the cytoplasm and the nucleus.
gsk-3	***gsk-3*** encodes the *C. elegans* glycogen synthase kinase ortholog; GSK-3 plays a role in regulating of the oxidative stress response pathway by phosphorylating SKN-1, thereby excluding it from intestinal nuclei.
hsf-1	***hsf-1*** encodes the *C. elegans* heat-shock transcription factor ortholog; HSF-1 functions as a transcriptional regulator of stress-induced gene expression whose activity is required for heat-shock and proteotoxicity response, larval development, innate immunity, and regulation of adult lifespan.
iftb-1	***iftb-1*** encodes the *C. elegans* ortholog of translation initiation factor 2 beta (eIF2beta); by homology, IFTB-1 is predicted to function in translation initiation and start codon recognition; loss of ***iftb-1*** activity in adult animals extends lifespan.
ist-1	***ist-1*** encodes a pleckstrin homology (PH) and phosphotyrosine binding (PTB) domain-containing insulin receptor substrate (IRS) homolog that negatively regulates lifespan and dauer development; IST-1 potentiates insulin-like signaling, although it is not absolutely required for such signaling under most conditions; in addition to acting through the AGE-1/PI3K branch of the insulin-

	like signaling pathway, IST-1 may also function in a parallel pathway to activate downstream protein-kinase Bs encoded by *akt-1* and *akt-2.*
jkk-1	***jkk-1*** encodes a member of the MAP kinase kinase superfamily that affects synaptic vesicle localization and is required in type-D motor neurons for normal locomotion; can function in the Hog1 MAP kinase pathway I in yeast as an activator of JNK and is expressed in most neurons
let-363	***let-363*** is an ortholog of human MTOR (mechanistic target of rapamycin (serine/threonine kinase)); ***let-363*** is involved in TOR signaling, determination of adult lifespan, growth, lipid metabolic process, positive regulation of translation, response to heat and translation; ***let-363*** is predicted to have 1-phosphatidylinositol-3-kinase activity, based on sequence information and is predicted to have drug binding activity, based on protein domain information; ***let-363*** is expressed widely; ***let-363*** is localized to the TORC1 complex, the TORC2 complex and the cytoplasm.
lin-2	***lin-2*** encodes a protein belonging to the membrane associated guanylate kinase (MAGUK) family, with several domains (L27, PDZ, SH3, and guanylate kinase) thought to assemble specific multiprotein complexes in particular regions of the cell; in vivo, LIN-2 is required for the correct localization of LET-23 (and, presumably, other membrane proteins) to specific regions of the plasma membrane.
lin-7	***lin-7*** istranslational reporter fusion indicates that LIN-7 is expressed at intestinal cell junctions; expression in vulval epithelial cells is detected only upon overexpression via heat shock, and reveals localization primarily at the lateral cell junctions of the vulval precursor cells P5.p and P6.p
mdl-1	***mdl-1*** gfp promoter fusions are expressed in a number of different tissues, including the posterior intestine, anterior and ventral cord neurons, pharyngeal and body wall muscles, somatic gonad precursors, and hypodermal cells; yeast one-hybrid and ChIP experiments indicate that DAF-3/Smad can bind the ***mdl-1*** promoter; in addition, ***mdl-1*** pharyngeal expression is specifically increased in *daf-3*(RNAi) animals, suggesting that DAF-3 directly negatively regulates mdl-1 transcription in pharyngeal tissue during dauer formation.
med-2	***med-2*** gene encodes a GATA-type transcription factor that is an immediate target of maternal SKN-1, and that participates in specifying the mesendoderm.
med-1	***med-1*** encodes a GATA-type transcription factor; *med-1* is an immediate target of maternal SKN-1 and, in the early embryo, participates in specifying the mesendoderm; MED-1 functions upstream of the END-1 and END-3 GATA-type transcription factors in endodermal cell fate specification.
mek-1	***mek-1*** encodes a MAP kinase kinase (MAPKK) that is involved in the stress response to heavy metals and starvation, and that has the highest homology to mammalian MKK7.
mpk-1	***mpk-1*** affect LET-60(Ras)-mediated induction of vulval cell fates, larval viability, morphology of the male spicules; ***mpk-1*** acts in combination with ***mek-2*** to permit germ cell exit from the pachytene stage of first meiotic prophase; MPK-1 activation is temporally/spatially dynamic compared to relatively constant levels of total MPK-1.

rps-6	***rps-6*** encodes a small (40S) ribosomal subunit S6 protein; loss of *rps-6* activity in adult animals extends lifespan.
sem-5	***sem-5*** acts downstream of the LET-23 epidermal growth factor receptor to negatively regulate RAS-, MAP-, and IP-3-, mediated signal transduction; a *sem-5* yfp promoter fusion is expressed in many cells throughout development, including the hypodermis, intestine, neurons, body wall muscles, and vulval precursor cells.
sek-1	***sek-1*** encodes a MAPKK (Mitogen-Activated Protein Kinase Kinase); SEK-1 functions in the p38 MAPK cascade that regulates innate immunity; SEK-1 has MAPKK activity; SEK-1 can activate both JNK-1 and PMK-1 in the yeast Hog pathway.
sgk-1	***sgk-1*** activity is required for normal egg laying, generation time, stress response, and adult life span; SGK-1 forms a complex with the AKT kinases with which it functions in parallel to mediate certain aspects of DAF-2/insulin-signaling; SGK-1 phosphorylates DAF-16 in vitro in a manner strictly dependent upon *pdk-1* which encodes a 3-phosphoinositide-dependent kinase.
smg-1	***smg-1*** functions as a key component of the nonsense-mediated mRNA decay (NMD) pathway that identifies and degrades mRNAs that contain premature stop codons; in addition, and partially in parallel to DAF-2-insulin signaling, SMG-1 regulates adult lifespan and the response to oxidative stress.
sod-3	***sod-3*** encodes an iron/manganese superoxide dismutase, predicted to be mitochondrial, that might defend against oxidative stress and promote normal lifespan; ***sod-3*** mRNA levels are diminished by mutation of *daf-16* and chromatin immunoprecipitation (ChIP) studies demonstrate that DAF-16 can directly bind the ***sod-3*** promoter; heterologously expressed SOD-3 in *E. coli* protects against methyl viologen-induced oxidative stress.
tir-1	***tir-1*** is required for innate immunity, as loss of *tir-1* activity results in increased susceptibility to infection; *tir-1* also functions in a lateral signaling pathway that specifies neuronal identity and is required for proper localization of NSY-1/MAPKKK to post-synaptic regions.
unc-14	***unc-14*** activity is required for axonogenesis (neurite outgrowth and axonal transport) and sex myoblast migration.
vhl-1	***vhl-1*** promotes the ubiquitination and degradation of the *hif-1* hypoxic response transcription factor; ***vhl-1*** and *hif-1* act to modulate life span and proteotoxicity, ***vhl-1*** mutants live longer compared to wild-type, by a mechanism separate from dietary restriction and insulin signaling; ***vhl-1*** may also have a *hif-1* independent function related to the extracellular matrix.

Table C1. This table represents all major genes that are known to have an effect on lifespan, nutrient signalling and a stress response.

CHROM	POSITION	REF	ALT	QUAL	VARIATION		VARIATION
III	2995523	T	G	210	WBVar01260924	JU1926 I, others T	WBVar01252910
III	2996977	A	C	210	WBVar01260925	exonic	low confidence
III	2997863	TAAAAAAA	TAAAAAA	68	low confidence	exonic	low confidence
III	2997962	A	T	210	WBVar01260927	intron	WBVar01406961
III	2998155	A	C	210	WBVar01260928	intron	Same as WBVar01252923
III	2998394	CAAAAAAAAA	CAAAAAAAAAA	53	low confidence	intron	WBVar00014613
III	2998509	T	A	212	WBVar01260930	intron	WBVar01252925
III	2999405	A	T	63	low confidence	5' UTR	WBVar01563160
III	2999707	A	G	210	WBVar01260932	JU1926 T, others I	WBVar00556754
III	3001857	G	T	210	WBVar01260935	intron	WBVar01393688

Table D1 Polymorphic Variation.
This table represents ten polymorphic variations between JU1511 and other parental strains in daf-2 gene. The result shows there were no non-synonimous changes in daf-2 gene and there is no expression difference in DAF-2 within the RILs.

APPENDIX D

Appendix E

Strain	Collected	Region	Sampled by
DL238	USA	Eugene	Ailion
EG4347	USA	Manuka	Knapp
JU1400	Spain	Sevilla	Felix
JU1401	Spain	Carmona	Felix
JU1411	Spain	Carmona	Felix
JU1416	Spain	Carmona	Felix
JU1511	France	Orsai	Felix
JU1516	France	Orsai	Felix
JU1522	France	Orsai	Felix
JU1581	France	Orsai	Felix
JU1920	France	Santeuil	Felix
JU1923	France	Santeuil	Felix
JU1926	France	Santeuil	Felix
JU1927	France	Santeuil	Felix
JU1931	France	Santeuil	Felix
JU1933	France	Santeuil	Felix
JU1936	France	Santeuil	Felix
JU1937	France	Orsai	Felix
JU1938	France	Orsai	Felix
JU1941	France	Orsai	Felix
JU1943	France	Orsai	Felix
JU1946	France	Orsai	Felix
JU1947	France	Orsai	Felix
JU1948	France	Orsai	Felix
JU1949	France	Orsai	Felix
JU323	France	Merlet	Felix
JU345	France	Merlet	Felix
JU393	France	Hermanville	Barriere
JU778	Portugal	Lisbon	Felix
MY23	GER	Roxel	Shulenburg
WN2002	Holand	Wageningen	Riksen
WN2003	Holand	Wageningen	Riksen

Table E1. Wild isolates. This table lists all the wild isolates used in this study, country of location and person who collected the samples.

APPENDIX F

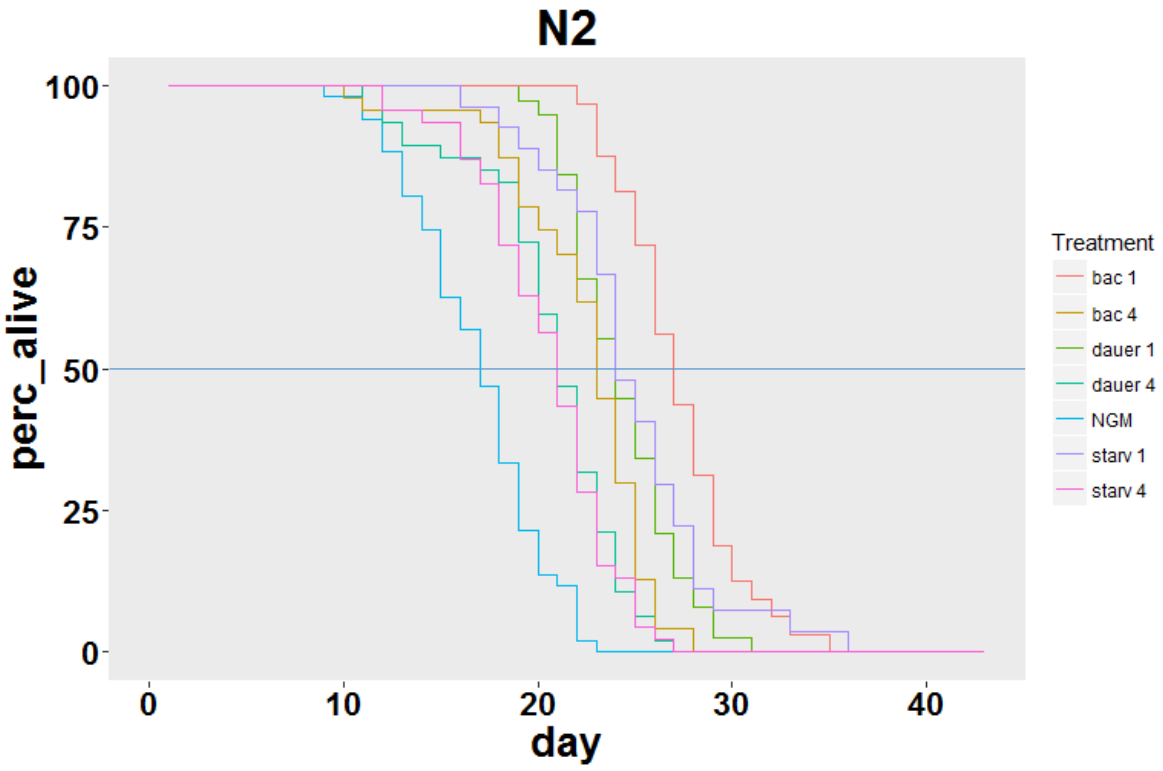


Figure F1. Mean lifespan correlates with 50% survival rate in N2 ±1 day.

Treatments	Mean lifespan Days	50% survial Days	Difference Days
Control	16.8±3.8	17	0.2
Dauer 1	24.1±2.8	24	0.1
Dauer 4	21.0±3.8	21	0
Bac Dil 1	27.2±3.1	27	0.2
Bac Dil 4	22.2±3.7	23	0.8
Starv 1	24.5±4.3	24	0.5
Starv 4	20.4±3.5	21	0.6

Table F2. Mean lifespan correlates with 50% survival rate in N2 ±1 day.

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